

THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Short-term adaptation of *S. cerevisiae* to lignocellulosic inhibitors

Underlying metabolic and physiological changes

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Gothenburg, Sweden 2021

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Cover: Schematic representation of a robust and a stressed cell.

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“In the middle of difficulty lies opportunity”

Albert Einstein

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ABSTRACT

The limited tolerance of *Saccharomyces cerevisiae* (budding yeast) to inhibitors present in lignocellulosic hydrolysates is a major challenge in second-generation bioethanol production. Short-term adaptation of the yeast to lignocellulosic hydrolysates during cell propagation has been shown to improve its tolerance, and thus its performance in lignocellulose fermentation. The overall aim of this thesis was to identify molecular and physiological changes during short-term adaptation.

In order to facilitate testing of *S. cerevisiae* physiology in lignocellulosic hydrolysate, a high-throughput methodology for the analysis of yeast strains in dark medium was developed. This methodology allows for monitoring of both aerobic and anaerobic growth of yeast in medium containing different hydrolysates at high reproducibility. The effect that individual nutrient components during propagation, rather than fermentation, has on lignocellulose fermentation performance is lacking. A high-throughput screening of certain vitamins, trace metals and nitrogen sources was performed. It was found that adding a mixture of pyridoxine, thiamine, and biotin to unadapted propagation cultures improved cell growth and ethanol yields during fermentation in wheat straw hydrolysate. Supplementing the propagation medium with nutrients in combination with short-term adaptation was thus demonstrated to be a promising strategy to improve the efficiency of industrial lignocellulosic fermentation.

Different *S. cerevisiae* strain backgrounds are used in the production of a suitable second-generation bioethanol host. In order to facilitate application of results obtained in laboratory experiments it is important to know whether short-term adaptation affects different strains differently. The physiology of two industrial *S. cerevisiae* strains were investigated while being short-term adapted. During propagation, fed with a hydrolysate containing feed, ethanol accumulation was observed for strain CR01 but not for KE6-12. Additionally, a larger increase in specific ethanol productivity for CR01 was observed than for KE6-12. Thus, short-term adaptation was found to affect *S. cerevisiae* physiology differently depending on strain background. To gain a more complete insight into the metabolic changes that *S. cerevisiae* experiences during short-term adaptation, RNA sequencing was performed on a time-series of samples taken from propagation cultures undergoing short-term adaptation. Expression data was compared to a non-adapted control using differential gene expression analysis. Results demonstrate, among others, an interesting role for multidrug proton antiporters *YHK8* and *FLR1* in the process of short-term adaptation.

Keywords: second generation bioethanol, short-term adaptation, lignocellulosic inhibitor tolerance, differential gene expression, industrial yeast strains.

Preface

This doctoral thesis fulfils the requirements for a PhD degree at the Department of Biology and Biological Engineering, Division of Industrial Biotechnology, Chalmers University of Technology, Sweden. The work presented in this thesis was performed between 2016 and 2021 and was funded by the Swedish Energy Agency (project nr 41252-1). The project was a collaboration between Chalmers, Lund University and Taurus Energy. The work was carried out at the Division of Industrial Biotechnology at Chalmers University of Technology, under the supervision of Professor Lisbeth Olsson and Associate Professor Yvonne Nygård.

Propagation experiments at process development unit scale were performed in collaboration with Dr. Borbála Erdei and Dr. Mats Galbe, Senior Lecturer at Lund University, Lund, Sweden. Pretreatment of the hydrolysates used in this work were performed by Dr. Borbála Erdei and Dr. Mats Galbe as well. The industrial strains used in the work was supplied by Taurus Energy AB.

Marlous van Dijk
February, 2021

List of publications

This thesis is based on the work contained in the following papers and manuscripts.

- I **van Dijk M**, Trollmann I, Saraiva MAF, Brandão RL, Olsson L, Nygård Y (2020) Small scale screening of yeast strains enables high-throughput evaluation of performance in lignocellulose hydrolysates. *Bioresource Technology Reports* 11:100532. doi: 10.1016/j.biteb.2020.100532
- II **van Dijk M**, Mierke F, Nygård Y, Olsson L (2020) Nutrient-supplemented propagation of *Saccharomyces cerevisiae* improves its lignocellulose fermentation ability. *AMB Express* 10, 157. <https://doi.org/10.1186/s13568-020-01070-y>
- III **van Dijk M**, Erdei B, Galbe M, Nygård Y, Olsson L (2019) Strain-dependent variance in short-term adaptation effects of two xylose-fermenting strains of *Saccharomyces cerevisiae*. *Bioresource Technology* 292:121922. doi: 10.1016/j.biortech.2019.121922
- IV **van Dijk M**, Rugbjerg P, Nygård Y, Olsson L (2021) RNA sequencing reveals metabolic and regulatory changes leading to more robust fermentation performance during short-term adaptation of *Saccharomyces cerevisiae* to lignocellulosic inhibitors *Manuscript*

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Author's contributions

- Paper I:** MvD conceptualized the study together with IT and YN. IT performed all experiments in the development of the screening method. MvD performed experiments in validation of the screening method. Data analysis was done by IT and MvD. YN and MvD drafted the manuscript and all authors contributed with discussing and interpretation of data and in developing the manuscript to its final format.
- Paper II:** MvD conceptualized the study together with LO and YN. Experiments were performed, and data was processed by FM. Data analysis was done by MvD and FM. MvD drafted the manuscript and all authors contributed with discussing and interpretation of data and in developing the manuscript to its final format.
- Paper III:** MvD conceptualized the study together with LO and YN. MG and BE produced the lignocellulosic material and analyzed its content. MvD performed all experiments, metabolite analysis and viability screen. Data analysis was done by MvD. MvD drafted the manuscript and all authors contributed with discussing and interpretation of data and in developing the manuscript to its final format.
- Paper IV:** MvD conceptualized the study together with LO and YN. MvD performed all experiments, metabolite analysis and sample preparation of the RNA samples. YN performed the experiments with the strains with *YHK8* and *FLR1* overexpressed and deleted. RNA sequencing and data quality control analysis was performed by SNP & SEQ Technology Platform in Uppsala. PR and MvD performed the data analysis. MvD drafted the manuscript and all authors contributed with discussing and interpretation of data and in developing the manuscript to its final format.

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1. Introduction

Ever since its domestication roughly 6000 years ago (Hornsey 2013), mankind has employed the yeast *Saccharomyces cerevisiae* for the production of various foods and compounds. It is no coincidence that distinct civilizations all over the world started using this yeast to produce fermented beverages and bread. During its domestication *S. cerevisiae* has developed to an incredibly robust industrial host, meaning that it is capable of maintaining production efficiency under the harsh and stressful conditions encountered in industry (Basso et al. 2008; Lopes et al. 2016). For the processes to be economical, it is desirable to produce the desired product at high concentrations, which imposes a stress on the cell. *S. cerevisiae* has shown to tolerate high levels of ethanol and, as such, has been used extensively in bioethanol production (Ma and Liu 2010; Pereira et al. 2010a).

A source of stress that is becoming increasingly relevant as industrial processes move towards the use of renewable resources are lignocellulosic inhibitors (LCIs). LCIs are by-products formed during pretreatment of lignocellulosic biomass, such as wood and non-edible parts of crops (Palmqvist and Hahn-Hägerdal 2000; Klinke et al. 2004). Monomeric sugars are released from the recalcitrant polymers in plant cell walls during pretreatment and the proceeding enzymatic hydrolysis. Lignocellulosic biomass is a desirable replacement for refined sugars in the production of renewable products, such as bioethanol. Refined sugars are sourced primarily from sugar cane in Brazil and corn production in North America and processes that use them as substrate are referred to as first-generation bioprocesses (Dutta et al. 2014). A concern that was levied against first-generation bioprocesses is that the use of refined sugars raises ethical issues over land use for the cultivation (Havlík et al. 2011; Rulli et al. 2016). The use of lignocellulosic biomass as a substrate is what is referred to as a second-generation bioprocess (Manochio et al. 2017).

LCIs generally encompass weak acids, furans and phenolics that each have their individual inhibitory effects on the physiology of *S. cerevisiae*, but also have been shown to act synergistically with each other (Oliva et al. 2006; Ding et al. 2011; Cunha et al. 2019).

Among other things, LCIs decrease cellular growth, viability and enzyme activity, leading to poor process economics as reviewed by Palmqvist and Hahn-Hägerdal (2000) and Galbe and Zacchi (2007). Presence of LCIs in the medium leads to prolonged lag phase and decreased specific growth rates for *S. cerevisiae* as exemplified in Figure 1.1. Therefore, it is important that the mechanisms underlying inhibition are studied in the complex LCI mixtures found in industrially relevant hydrolysates.

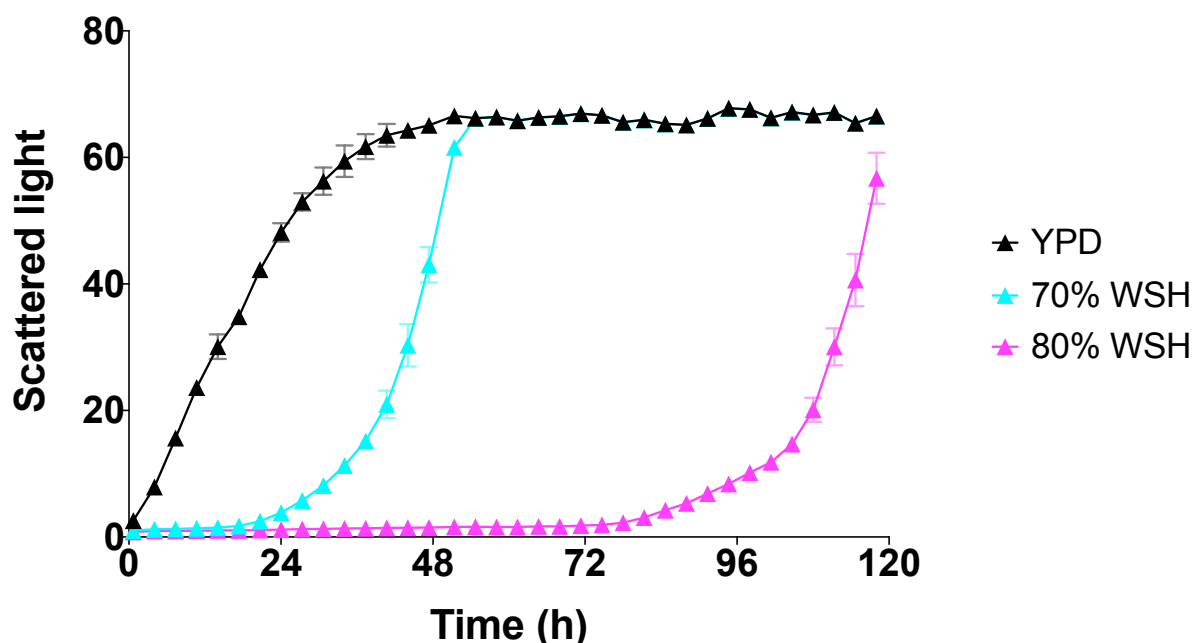


Figure 1.1. Growth inhibition of *S. cerevisiae* due to the presence of lignocellulosic inhibitors, coming from wheat straw hydrolysate (WSH), in the medium. The experiment was performed in aerobic microbioreactors. Control cultures were grown in complex medium containing yeast extract, peptone and dextrose (YPD). This figure was adapted from **Paper I, Figure 1A**.

In this thesis, I chose to work with hydrolysate as it would be used in industry as opposed to a synthetic mixture of LCIs that sometimes is used in lab experiments. An additional challenge in improving *S. cerevisiae* robustness to LCIs is that lignocellulosic biomass inherently varies in composition seasonally. During pretreatment and enzymatic hydrolysis, different profiles of inhibitors are produced depending on when the raw biomass was harvested (Bunnell et al. 2013; Greenhalf et al. 2013), which raw biomass is used (Klinke et al. 2004; Almeida et al. 2007) as well as which methods of pretreatment are used (Chundawat et al. 2010). Even when using the same pretreatment method and using raw biomass from the same harvest, batch-to-batch variability is observed in the inhibitor profiles produced (Table 3.3.1). This variable nature of the substrate means that using evolutionary or genetic engineering, where a host's production efficiency is optimized for a single environmental condition, is of limited use when trying to improve the efficiency of hydrolysate fermentation towards a broad range of hydrolysates.

It has been shown that exposing *S. cerevisiae* to a non-lethal amount of stress confers protection against subsequent exposure to the same, or other types of stresses that would otherwise have been lethal (Alkasrawi et al. 2006; Nielsen et al. 2015; **paper III**). Short-

term adaptation is the process where *S. cerevisiae* is propagated in the presence of a dilute concentration of the same lignocellulosic hydrolysate which is also used in the subsequent fermentation. It has been shown to improve viability, biomass yield, and fermentation capacity of *S. cerevisiae* during subsequent hydrolysate fermentation. It is a potential solution to mitigate LCI toxicity that is more flexible than classical evolutionary or genetic engineering of *S. cerevisiae* strains. Propagation is an existing operational step in bioethanol production and applying short-term adaptation does not require any major changes to be made in the bioethanol production process. However, knowledge of the mechanisms underlying short-term adaptation is currently lacking. Therefore, the aim of my thesis was to understand these mechanisms better. To realize this, several research questions were identified and addressed. These questions as well as the approach that was taken to answer them are stated below.

1. How can the throughput capacity of the screening of growth of *S. cerevisiae* in dark hydrolysate medium be increased?

Advances in strain engineering greatly decreased the time necessary for strain construction and even allow for construction of multiple strains in parallel as reviewed by (Petzold et al. 2015). Screening of newly constructed strains in relevant growth media needs to match pace with strain construction to avoid becoming a bottleneck in the design-build-test-learn cycle. Currently, screening of newly designed strains with potential increased LCI tolerance generally takes place in defined synthetic medium due to the drawbacks that screening with hydrolysate-containing medium has. Absorption as a measure for cell density is incompatible with the dark color of hydrolysates. Therefore, screening *S. cerevisiae* growth in dark hydrolysate medium requires time-consuming techniques to be used such as counting of colony-forming-units or cell counting using a Neubauer chamber. This also means that multi-well cultivation platforms that use absorption to determine cell density, which most commercially available platforms do, are not suitable for screening growth of *S. cerevisiae* in dark hydrolysate medium at high throughput. The Biolector platform is a multi-well cultivation system that uses scattered light instead of absorption to measure cell density. In **paper I**, the development of a method to screen growth of a variety of *S. cerevisiae* strains in dark hydrolysate medium in a high throughput manner was developed. The method was used, among other things, to monitor growth of *S. cerevisiae* in dark hydrolysate fermentation medium after short-term adaptation to dilute hydrolysate concentrations during propagation.

2. To what extent does nutrient addition during the propagation of *S. cerevisiae* improve its subsequent hydrolysate fermentation performance?

Process conditions during propagation are known to determine the productivity of the inoculum that is produced. Addition of nutrients to the fermentation medium is known to improve productivity (Jørgensen 2009; Xiros and Olsson 2014; Kelbert et al. 2015). Supplementing nutrients to the propagation medium and evaluating subsequent fermentation productivity could inform us what cellular processes are important to produce an inoculum with better hydrolysate fermentation performance. Using the Biolector cultivation method, I screened growth of propagation cultures with addition of nutrients known to improve LCI

tolerance during fermentation (**paper II**). Subsequently, we evaluated whether the addition of those nutrients only to the propagation medium leads to improved fermentation performance of the propagated cells.

3. To what extent does addition of certain nutrients to the propagation of *S. cerevisiae*, that are also being subjected to short-term adaptation, improve its subsequent lignocellulose fermentation performance?

Two approaches to improve lignocellulosic fermentation were tested; (i) using short-term adaptation or (ii) nutrient addition during propagation, after which the two methods were combined. If the combination led to further improvements, this would be an indication that cellular processes that improved productivity by nutrient addition, at least partly, have additional function in robustness compared with those that improved productivity by short-term adaptation. Using the Biolector cultivation method, we screened growth of propagation cultures, but now with addition of both nutrients and a non-lethal concentration of hydrolysate (**paper II**). We also evaluated subsequent hydrolysate fermentation performance of the propagated strains.

4. Is the effect of short-term adaptation on lignocellulose fermentation performance of *S. cerevisiae* strain dependent?

There are many different strains of *S. cerevisiae* that are being used in industry and in research. Among them there is an array of differences in their genetic backgrounds that result in slightly different phenotypes. We evaluated physiological parameters, that can be considered indicators of increased LCI robustness, of two closely related *S. cerevisiae* strains during short-term adaptation (**paper III**). Both *S. cerevisiae* strains were engineered for xylose consumption and LCI robustness and originate from the same strain background. Parameters that were investigated were intracellular trehalose and glycogen content (during propagation), cell viability (during propagation and fermentation), and ethanol productivity (during subsequent hydrolysate fermentations).

5. Which genes or metabolic pathways are differentially expressed during short-term adaptation of *S. cerevisiae*?

Transcriptomic studies investigating mechanisms of LCI tolerance or toxicity have generally focused on the fermentation step where inhibitor concentrations are high and therefore a strong stress response is commonly observed. The metabolic response of *S. cerevisiae* to LCIs during propagation is likely markedly different due to the availability of molecular oxygen. Such studies are often carried out in shake flasks, meaning that process conditions are not well controlled and only a single time-point can be captured (Chen et al. 2016; Brandt et al. 2019). Moreover, such transcriptomics studies have mostly focused on addition of a single inhibitor (Dong et al. 2017; Liu and Ma 2020) or a synthetic mixture of a few inhibitors (Bajwa et al. 2013; Thompson et al. 2016). Investigation using real hydrolysates are uncommon as reviewed by (Cunha et al. 2019). It is challenging to detect all compounds present in lignocellulosic hydrolysate because of its complex nature and therefore difficult to design realistic model systems. Therefore, it is crucial to study robustness mechanisms of *S. cerevisiae* in the presence of real hydrolysates. RNA sequencing was performed on a time

series of samples taken during the feed phase of *S. cerevisiae* propagation that was fed with and without hydrolysate (**paper IV**) in order to identify possible genes or metabolic pathways that are involved in the short-term adaptation process.

Chapter 2 will introduce the fundamental aspects of central carbon metabolism and energetics in *S. cerevisiae*, it will explain the phenomenon of aerobic fermentation and it describes different methods of cultivation and their characteristics. **Chapter 3** explains the procedure for industrial propagation of *S. cerevisiae* as well as two substrates that are of importance in the cellulosic bioethanol production process: molasses, commonly used as carbon source for *S. cerevisiae* propagation and lignocellulosic hydrolysate, used as carbon source during fermentation to produce ethanol. **Chapter 4** describes different types of stress and how they affect *S. cerevisiae* metabolism. **Chapter 5** introduces several methods to combat the inhibition of *S. cerevisiae*. This chapter also describes the development of a high-throughput cultivation method for growth in hydrolysate. Findings regarding nutrient supplementation during propagation, strain dependent differences in physiological response to short-term adaptation as well as differential expression during short-term adaptation are also presented.

2. Yeast metabolism

In a metabolic pathway, the substrate for each reaction is the product of a previous reaction. As per the first law of thermodynamics, the rate at which the substrate is supplied is equal to the rate at which it is converted (Nelson and Cox 2008, p 487). This means that in multistep pathways such as glycolysis, the rates of many reactions rise and fall with substrate concentration. The metabolism is tightly regulated to maintain a balanced metabolic network and to prevent unnecessary energetic losses.

2.1 Central carbon metabolism

The carbon flux entering the cell is distributed between catabolism and anabolism. Glucose is the preferred carbon and energy source for *S. cerevisiae*, but it can utilize other carbon containing compounds as well. Catabolism involves the oxidation of the substrate entering the cell and results in production of adenosine triphosphate (ATP), nicotinamide adenine dinucleotide (NADH) and nicotinamide adenine dinucleotide phosphate (NADPH) which are subsequently used to drive other important cellular functions (Figure 2.1.1). Many reactions in the cell are oxidation – reduction reactions that require specialized electron carriers, also called redox co-factors, primarily NAD^+ and NADP^+ (Nelson and Cox 2008, p 487). Reduced electron carriers NADH and NADPH are products of catabolism and can donate electrons in processes that generate ATP or drive reductive steps in biosynthetic pathways.

Regulatory mechanisms maintain nearly constant levels of ATP, NADH and NADPH in cells, while matching the use or production of glucose to the cell's changing needs. Pyruvate and acetyl-CoA are also maintained at steady-state concentrations as they are important regulatory points in metabolism. Anabolism, or biosynthesis, is the production of cellular components necessary for growth and cell division. In these processes, substrates are converted to more complex molecules such as nucleic acids, proteins, polysaccharides, lipids, etc. Anabolic reactions generally require the input of ATP, NADH and NADPH. As

a consequence of the required balance of ATP, NADH and NADPH, rates of catabolism and biosynthesis will be balanced to meet the cellular needs.

Chemical energy, stored in the forms of ATP and NAD(P)H, is not just used for biosynthesis but is used in processes that keep the cell functioning properly including repair of cell damage, protein turnover and pH homeostasis (van Bodegom 2007). These processes are grouped under the term maintenance. The energy requirements for maintenance under optimal, laboratory conditions is a small fraction of the overall amount of energy that is produced and is considered to be strain-dependent (Verduyn 1991; van Bodegom 2007). However, under stressful conditions the maintenance energy requirements increase, thereby diverting energy away from growth. An example of balancing that is important within maintenance processes is protein turnover. Rapid turnover of proteins (synthesis followed by degradation) can be energetically expensive, but proteins with a short half-life can reach new steady state levels much faster than those with a long half-life, and the benefit of this quick responsiveness may outweigh the cost to the cell.

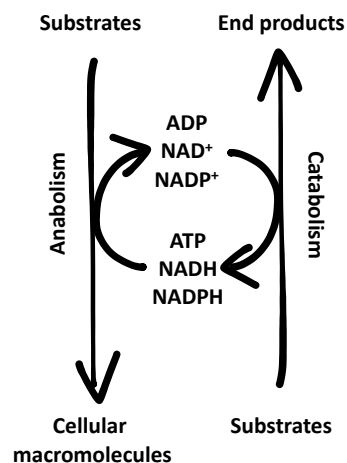


Figure 2.1.1. Schematic representation of the relationships between energy and redox co-factors in anabolism and catabolism. Substrates that are taken up by the cell are divided between catabolism, yielding oxidized, energy depleted end products, or anabolism, yielding cellular macromolecules such as nucleic acids, proteins, polysaccharides or lipids. Image adapted from (Nelson and Cox 2008, p 487).

2.2 Aerobic fermentation

An important parameter when quantifying growth efficiency is the cell mass yield ($g_{\text{cell mass}} g_{\text{glucose}}^{-1}$). The theoretical cell mass yield is not equal to the complete incorporation of carbon from the substrate into cell mass. During anabolic reactions, a net production of CO_2 always occurs and as such, the carbon conversion is always lower than 100% (Verduyn 1991). The fraction of carbon lost in CO_2 has been reported to be roughly 10% for growth on glucose and up to 29% for other carbon sources (Gommers et al. 1988). Another factor that influences the theoretical cell mass yield is that biosynthesis requires NADPH. The mitochondrial

NADP⁺ dependent isocitrate dehydrogenase can supply NADPH for biosynthesis, but is not capable of supplying sufficient NADPH to maximize the cell mass yield (Bruinenberg et al. 1983a). Studies in *Candida utilis* showed that roughly 7% of the carbon source is oxidized in the pentose phosphate pathway, another source of NADPH in metabolism (Bruinenberg et al. 1983b).

The cell mass yield was shown to be related to the efficiency of ATP formation in catabolism (Bauchop and Elsdon 1960). *S. cerevisiae* has two main pathways of ATP production from glucose, respiration and fermentation as reviewed by, among others, Pfeiffer and Morley (2014). Both pathways start with glycolysis and split at pyruvate. Respiration (i.e., complete degradation of glucose to CO₂ through glycolysis and the citric acid cycle) theoretically yields 38 molecules of ATP per molecule of glucose. Because *S. cerevisiae* uses a single subunit, non-proton translocating NADH dehydrogenase, the phosphate/oxygen ratio (P/O ratio) is low (estimated to be 1.2; van Gulik and Heijnen 1995; De Kok et al. 2012) and the effective yield of ATP per molecule of glucose is closer to 18 than 38 (Pfeiffer and Morley 2014). Regardless, respiration is an energetically optimal way to utilize glucose. However, *S. cerevisiae* is known to catabolize glucose to ethanol in the presence of oxygen, yielding only 2 molecules of ATP per molecule of glucose. This phenomenon of aerobic fermentation is also called the Crabtree effect and occurs when glucose concentration in the medium is above roughly 0.1 g L⁻¹ (depending on strain and growth conditions; Crabtree 1929; Van Dijken and Scheffers 1986). Aerobic fermentation generally occurs in aerobic batch cultivations of *S. cerevisiae*. During aerobic fermentation, transcription of genes involved in respiration, in gluconeogenesis and in use of carbon sources other than glucose are repressed in a process referred to as glucose repression (Klein et al. 1998; Gancedo 1998; ROLLAND et al. 2002). Glucose sensing and regulation of glucose uptake is performed by the Snf3/Rgt2 signaling pathway (Kaniak et al. 2004). The Snf1 protein kinase plays a dual role in glucose repression. High glucose concentrations inactivate Snf1, leaving Mig1 non-phosphorylated and thus capable of translocating into the nucleus to repress genes (Gancedo 1998; Hedbacker 2008). However, when glucose concentrations drop below 0.2%, Snf1 is activated allowing for phosphorylation of Mig1, thereby inactivating it and relieving glucose repression (Piškur and Compagno 2014). After glucose repression is alleviated in an aerobic batch culture, the accumulated ethanol resulting from aerobic fermentation can be consumed by *S. cerevisiae* provided that molecular oxygen is present. This shift in metabolism from fermentation to respiration is referred to as the diauxic shift and is regulated by several signaling pathways and transcription factors as reviewed by (Galdieri et al. 2010).

Cell mass yields on glucose vary due to the difference in ATP-yields of respiratory, fermentative, and respiro-fermentative metabolism. Empirically determined values for the cell mass yields of *S. cerevisiae* are 0.52 g_{cell mass} g_{glucose}⁻¹ under fully respiratory metabolism (D = 0.10 h⁻¹; Pronk et al. 1994), 0.1 g_{cell mass} g_{glucose}⁻¹ under anaerobic fermentative metabolism (D = 0.10 h⁻¹; Verduyn et al. 1990) and 0.16 g_{cell mass} g_{glucose}⁻¹ under respiro-fermentative metabolism (D = 0.47 h⁻¹; Postma et al. 1989). However, the cell mass yield differs with changes in cell composition, transport of the carbon source, P/O-ratio and maintenance processes (Verduyn 1991).

2.3 Modes of cultivation

Batch cultivation is the simplest method of *S. cerevisiae* cultivation. It is a mode of cultivation where all medium components are present before inoculation and no additional nutrients are added to the medium. During the exponential growth phase, the culture generally grows at its maximum specific growth rate (μ_{\max}) because of the excess glucose concentration at the start of a batch cultivation. Therefore, batch cultivations are suitable for determining μ_{\max} as well as for preliminary screening of different strains or experimental conditions. As such batch cultivation was used in **papers I** and **II**. Batch cultivation was also used for fermentation in **papers III** and **IV** because this is an industrially relevant cultivation method and because ethanol productivity, the parameter that was used for evaluation in these experiments, can be reliably measured in a batch (Figure 2.3.1).

A disadvantage of batch cultivations is that the experimental conditions are not constant. Concentrations of nutrients decline throughout a batch whereas product concentrations increase. Therefore, conditions during a batch experiment are far from being a steady-state environment and more precise quantification of *S. cerevisiae* physiology is not possible. Another disadvantage of batch cultivation, from an industrial perspective, is that yeast grows using respiro-fermentative metabolism and therefore cell mass yields are far below optimal values.

Chemostat, or continuous, cultivation is a method of cultivation where, after an initial batch phase, the culture is fed with nutrients while culture broth is pumped out of the reactor at the same flow rate as the feed. Using this mode of cultivation, a pseudo-steady state of the metabolism sets in after a few volume changes and it allows for much more precise determination of specific rates of production and consumption of intracellular metabolites. Chemostat cultivation of *S. cerevisiae* using a hydrolysate medium was attempted for the work presented in this thesis (data not shown). The main reason these chemostats were attempted was to identify a critical specific growth rate (discussed in **Chapter 3.2**) for growth in the presence of hydrolysate. However, when performing the chemostats with hydrolysate medium, cultures never reached the pseudo-steady state required for sampling. Rather, the imposed dilution rate (i.e., the specific growth rate imposed on the culture) of 0.05 h^{-1} was too high and the culture washed out of the bioreactor. This result shows the considerable degree of inhibition that the hydrolysate imposes on the cells. Due to equipment limitations, chemostat cultivation below a dilution rate of 0.05 h^{-1} were not attempted.

Cultivation methods used in this thesis

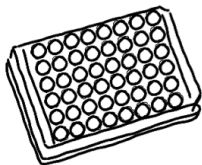
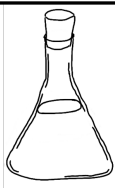
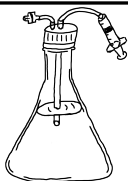

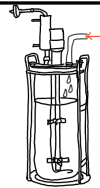
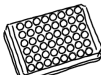
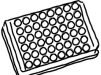


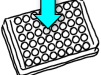


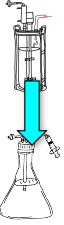
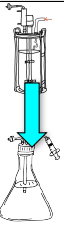
					
	Multi-well plate	Aerobic shake flask*	Anaerobic shake flask	Batch reactor	Fed-batch reactor
Paper I	  	Figures 1A, 1C and 2A (1 mL working volume): - Batch - Aerobic		- Growth at μ_{\max} - <u>Respiro-fermentative</u> metabolism	
		Figures 1D, 1E and 2B (1 mL working volume): - Batch - Anaerobic		- Growth at μ_{\max} - <u>Fermentative</u> metabolism	
		Figures 1B (100 mL working volume): - Batch - Aerobic		- Growth at μ_{\max} - <u>Respiro-fermentative</u> metabolism	
Paper II	   	Figures 1 and 2 and Table 4: Propagation (50 mL working volume): - Batch - Aerobic Fermentation (1 mL working volume): - Batch - Anaerobic		- Growth at μ_{\max} - <u>Respiro-fermentative</u> metabolism - Growth at μ_{\max} - <u>Fermentative</u> metabolism	
		Table 5: Propagation (50 mL working volume): - Batch - Aerobic Fermentation (200 mL working volume): - Batch - Anaerobic		- Growth at μ_{\max} - <u>Respiro-fermentative</u> metabolism - Growth at μ_{\max} - <u>Fermentative</u> metabolism	
Paper III		Figures 1 – 3: Propagation (500 - 1000 mL working volume): - Fed-batch - Aerobic Figures 4 – 6 and Table 1: Fermentation (200 mL working volume): - Batch - Anaerobic		- Growth at $\mu = 0.05 \text{ h}^{-1}$ - <u>Respiratory</u> metabolism (control) - <u>Respiro-fermentative</u> metabolism (hydrolysate-adapting) - Growth at μ_{\max} - <u>Fermentative</u> metabolism	
Paper IV		Figure 1: Propagation (500 - 1500 mL working volume): - Fed-batch - Aerobic Figure 2: Fermentation (200 mL working volume): - Batch - Anaerobic		- Growth at $\mu = 0.05 \text{ h}^{-1}$ - <u>Respiratory</u> metabolism (control) - <u>Respiro-fermentative</u> metabolism (hydrolysate-adapting) - Growth at μ_{\max} - <u>Fermentative</u> metabolism	

Figure 2.3.1. Different scales & methods for cultivation of *S. cerevisiae* that are used in this thesis. Using methods such as a multi-well plate or shake flask does not allow for feeding of nutrients and thus does not allow for control of the specific growth rate (μ). Using a bioreactor allows for feeding of nutrients and thus allows for a pre-determined μ , but also allows for better control of process parameters such as aeration and pH. Figures and tables referred to in this figure are located in the corresponding papers and not in this thesis chapter. *All experiments were started with an aerobic cultivation in shake flasks, inoculated with cells from a cryo-stock.

For industrial production of *S. cerevisiae*, the main reason why chemostat cultivation is not extensively used is the increased risk of contaminations as well as the laborious and complex nature of operations. However, it is vital to maximize cell mass yields for economics of the production process. In a fed-batch cultivation, the reactor is fed after the batch phase has finished but no culture broth is pumped out. Provided that the feed is substrate-limiting, this way, *S. cerevisiae* grows at a specific growth rate (μ) that is determined by the operator of the reactor. If the μ imposed on the culture is lower than a certain value, the culture can maintain full respiratory metabolism and thereby maximize cell mass yield.

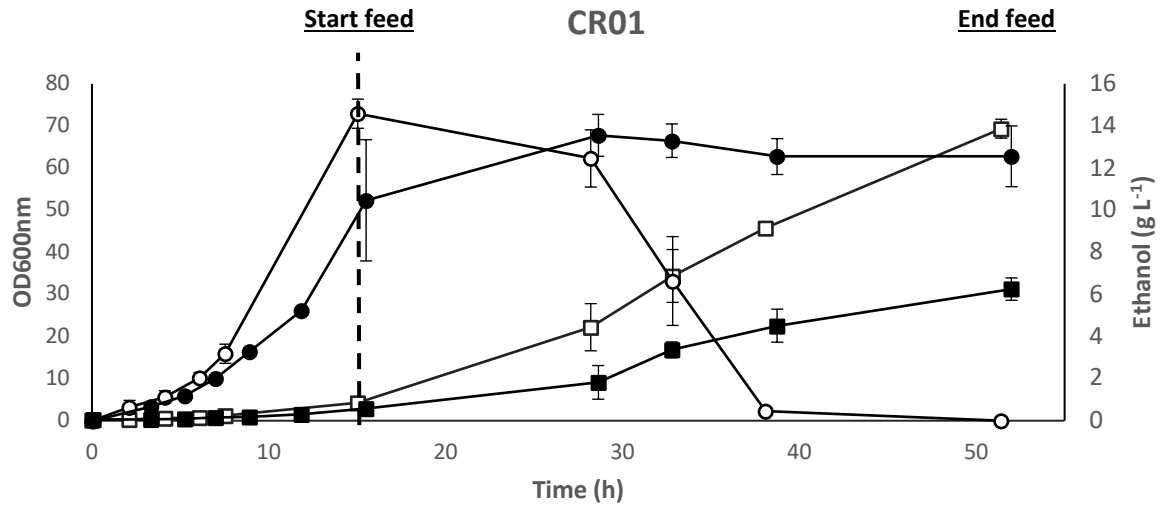


Figure 2.3.2. Fed-batch propagation profiles of *S. cerevisiae* strain CR01 fed with a hydrolysate solution (closed symbols) and with a molasses solution (open symbols). Depicted are optical density at 600 nm (OD_{600nm} ; squares) and ethanol concentration (circles). The dotted line indicates the start of the feed phase, which is preceded by a batch phase. Values given are the average of two biological replicates, and the error bars depict the standard error. Adapted from **paper III**, **Figure 1A**.

Due to its industrial relevance, fed-batch cultivation was used in **papers III** and **IV** for a more rigorous investigation of short-term adaptation during propagation (Figure 2.3.1). An example of a fed-batch cultivation profile is shown in Figure 2.3.2. The control cultivation (open symbols) shows a fed-batch profile typical for *S. cerevisiae*. During the batch phase, ethanol is accumulated in the medium due to the Crabtree effect, whereas during the feed phase the ethanol is consumed due to the low μ , allowing for a full respiratory metabolism. However, as discussed in **paper III**, the hydrolysate fed cultivation (closed symbols) did not consume ethanol during the feed phase. This was likely due to a lowering of the μ at which the Crabtree effect sets in because of the presence of hydrolysate. As mentioned above, equipment limitations did not allow for a $\mu < 0.05 \text{ h}^{-1}$ to be imposed on the system.

3. Industrial cultivation of *S. cerevisiae*

S. cerevisiae has been used in industrial fermentation processes for thousands of years for beverage fermentation and bread leavening purposes. Annual baker's yeast production for human food consumption is around 2 million metric tons globally (Attfield 1997; Hui et al. 2004). In recent years application of *S. cerevisiae* in industrial production has intensified. Modern industrial applications of *S. cerevisiae* are often for production of bio-based products replacing their petroleum counterparts. Some examples are production of cellulosic ethanol (Della-Bianca et al. 2014), lactic acid (Vink et al. 2004), succinic acid (Jansen and van Gulik 2014) and farnesene (Chandran et al. 2011). To ensure fast, reliable, and complete fermentation, modern industrial processes require large quantities of *S. cerevisiae*. Since the introduction of active dry yeast in Europe in the 1960's (Reed and Nagodawithana 1988), large scale *S. cerevisiae* propagation has been optimized to increase biomass yield and decrease production cost as reviewed by Gómez-Pastor et al. (2011). However, since then studies have reported that *S. cerevisiae* encounters a variety of stress factors during large scale propagation that can influence its fermentation performance significantly (Attfield 1997; Hong et al. 2010). Here, I discuss the process of propagation of *S. cerevisiae* at an industrial scale as well as two substrates that are important for second-generation bioethanol production. Molasses, commonly used as carbon source during propagation, is described as well as its nutritional value for *S. cerevisiae* cell mass production. Lignocellulosic hydrolysate, used as a substrate in the anaerobic ethanol fermentation, is described in terms of how it is produced and how its components affect *S. cerevisiae* metabolism. Hydrolysate is also used in the propagation when short-term adaptation is applied.

3.1 Propagation at industrial scale

The procedure for industrial *S. cerevisiae* propagation has been modeled after Baker's yeast propagation procedures, which is a multi-stage process in which culture volume is gradually scaled-up from shake flasks to bioreactors of increasing size (Figure 3.2.1.; Reed and Nagodawithana 1991). The process is designed in such a way to produce the vast quantities

of yeast required for fermentation. Scaling up propagation in a single (or fewer) step(s) would be inefficient because this would require high sugar concentrations in the medium leading to respiro-fermentative growth and thus decreased cell mass yields in addition to severe osmotic stress (Pérez-Torrado et al. 2009). *S. cerevisiae* propagation is generally carried out under continuous aeration because the presence of O₂ supports sterol production, required for efficient growth, but also because the presence of O₂ leads to higher biomass yields by preventing fermentation to ethanol provided glucose addition is controlled. The subsequent few stages of propagation are fed after the batch phase finishes ensuring a full respiratory metabolism (Figure 3.1.1).

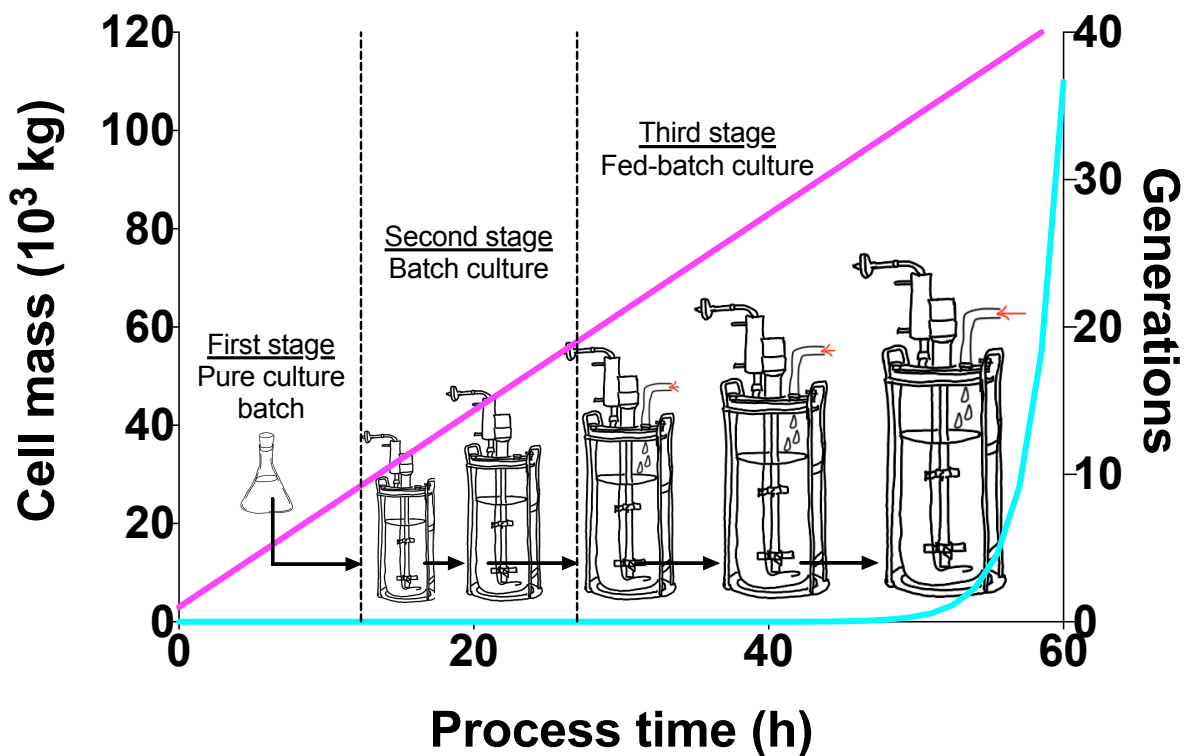


Figure 3.1.1. Schematic representation of industrial propagation of *S. cerevisiae* in a multi-stage process. In the first stage, pure cultures are grown, which are used to inoculate larger batch cultures in the second stage. In stage three, reactors are fed to obtain optimal cell mass yields. Reactor volume increases upon every re-inoculation step. *S. cerevisiae* cell mass produced throughout the process is shown in turquoise and generation numbers in magenta. Figure adapted from Reed and Nagodawithana (1991), Rose and Vijayalakshmi (1993) and Gómez-Pastor et al. (2011)

The multi-stage propagation process is necessary to obtain suitable quantities of cell mass for fermentation purposes. However, this sequential cultivation process introduces considerable generation numbers after the initial pure culture (Figure 3.1.1). The replicative lifespan is the number of doublings a mother cell can go through before it stops producing daughter cells. Depending on process conditions, laboratory strains of *S. cerevisiae* have been reported to have a mean replicative lifespan between 9 and 32 (Ashrafi et al. 1999; Powell et al. 2000; MASKELL et al. 2003; Qin and Lu 2006), whereas wild isolates were reported to have a slightly higher replicative lifespan of 22 – 44 (Qin and Lu 2006). Thus, at the end of the multi-stage propagation process, the culture has gone through one or more

rounds of complete turnover resulting in a cell population that is likely metabolically very different from the cell population in the first pure culture. It is therefore important to not only investigate *S. cerevisiae* propagation at an industrially relevant scale to identify possible scale-up effects, but it is similarly important to investigate the sequential stages of the process to identify effects from increased generation numbers.

As pentose fermentation is not as efficient as glucose fermentation, the required amount of yeast to pitch will be higher in second than in first generation bioethanol production. Also, the presence of inhibitors reduces the ethanol productivity, and a higher yeast pitch will be beneficial. Xiros and Olsson (2014) have investigated the effect of the size of the pitch on the fermentation performance in spruce hydrolysate. When a pitch of 3 g_{dry weight} L⁻¹ yeast was used, the maximum ethanol concentration was reached after 40 h; when using 2 g_{dry weight} L⁻¹ yeast, the maximum ethanol concentration was reached after 110 h and when using 1 g_{dry weight} L⁻¹ or 0,5 g_{dry weight} L⁻¹ yeast, the fermentation did not get started during the 110-h long experiment. Consequently, the need of a large yeast inoculum can be a major contributor to the ethanol production cost. Estimations have been made showing that it is likely that around 0.1-0.2 g L⁻¹ of cells is the maximum amount which a production process could afford to carry in terms of purchase cost (personal communication, Lisbeth Olsson). Since about 2-3 g L⁻¹ is needed for a robust fermentation a method for on-site yeast propagation is needed. It is essential to find robust protocols for propagation of cells, which are able to withstand the rather harsh conditions prevailing during fermentation of lignocellulosic hydrolysates. The demonstration of the beneficial effect of short-term adaptation is an important contribution to increase yeast efficiency and decrease the needed amount of cell mass.

Different scenarios are possible for the supply of cells to the process; (i) the cells are propagated from a strain stock over several steps, (ii) the yeast is bought from a yeast supplier and the cells are propagated in a propagation reactor before transferred to the fermentation step (iii) the yeast is bought from a yeast supplier and the fermentation step is started with an aeration period in which the cells are allowed to grow to sufficient levels, after which fermentation under anaerobic conditions proceeds (iv) the yeast is bought from a yeast supplier in large quantities and used without propagation. The three first scenarios have the best economical potential and using them allows the introduction of short-term adaptation using the same hydrolysate as in the fermentation step. We therefore attempted to gain information on the short-term adaptation in propagations that took place over a larger number of generations.

The propagation procedure was performed by starting with a batch seed in shake flask (working volume 100 mL), transferring to a batch seed in a bioreactor (working volume 1.5 L) and finally inoculating a fed-batch propagation culture (working volume batch: 10 L, feed: 5 L). The seed medium was defined synthetic medium as described by Verduyn et al. (1992), while the propagation medium consisted of molasses (2% glucose equivalent) supplemented with 2.3 g L⁻¹ urea, 3 g L⁻¹ potassium phosphate, 0.5 g L⁻¹ magnesium sulfate, and 0.033 mg L⁻¹ D-biotin. The propagation performance in 15L scale were very similar to the performance of the propagations in paper III (see Figure 2.3.2) and IV (Figure 1 (in

paper)). The outcome of the proceeding fermentation showed a clear positive effect of the short-term adaptation (Figure 3.1.2). The ethanol concentration after 48 h of fermentation was 24,3 g L⁻¹ in the fermentation using the adapted culture, whereas it was 20,6 g L⁻¹ in the non-adapted culture. While the glucose consumption was similar in the two cultures, there was a significant difference in the ability of utilizing xylose, 68 % of the xylose remained after 48 h of fermentation using the non-adapted culture, whereas only 41 % of the xylose remained when the adapted culture was used.

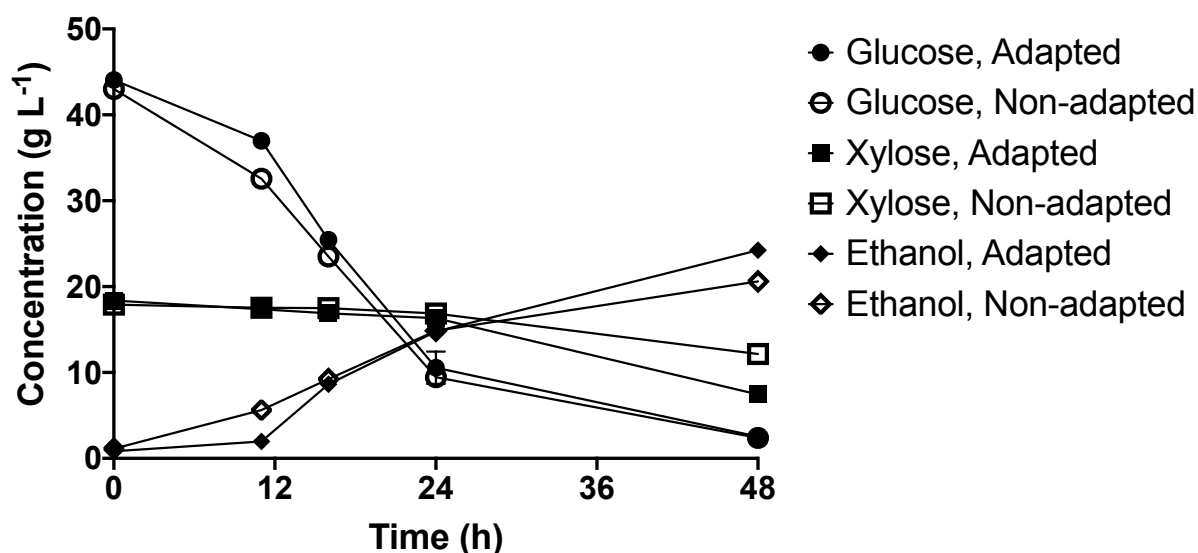


Figure 3.1.2. Fermentation profiles of short-term adapted (closed symbols) and non-adapted (open symbols) *S. cerevisiae* cultures in 70% wheat straw hydrolysate-containing medium (composition of hydrolysate: Table 3.3.1, last row).

The outcome of the fermentation using cultures from cultivations in 15 L scale with a higher number of generations did not lead to as pronounced difference between the adapted and non-adapted cells as the results from 1 L scale (paper III and IV). Further experiments would be required to elucidate which were the differences of the experiments in the different scales. It should be pointed out that the hydrolysates used were produced in a different batch (see Table 3.3.1, last row), and therefore its composition was slightly different. Work outside the thesis work will elucidate the transcriptional differences during propagation using different number of generations. The most important conclusion from these experiments is that short-term adaptation is scalable.

3.2 Substrate – Molasses

While laboratory propagation generally relies on glucose based medium, sugar cane or beet molasses is used as a carbon source in industrial *S. cerevisiae* propagation (Gómez-Pastor et al. 2011). Main reasons for this are that molasses is generally high in sucrose, which *S. cerevisiae* readily consumes, and that molasses is a by-product from the sugar industry with a relatively low value. One disadvantage of using molasses is that it contains herbicides, insecticides, fungicides, fertilizers and heavy metals which are known to inhibit *S. cerevisiae*.

growth (Pérez-Torrado 2004). A transcriptomic study comparing *S. cerevisiae* growth on molasses and synthetic medium confirmed the existence of toxic components in molasses, most notably by the induction of genes *FDH1* and *FDH2* for the detoxification of formate (Shima et al. 2005). *FDH1* and *FDH2* were found to be 14.6-fold and 14.8-fold upregulated, respectively, in yeast grown on molasses compared with growth on synthetic medium. Cells grown on molasses had also accumulated 1.8-fold more trehalose than cells grown on synthetic medium. Trehalose has been shown to accumulate in response to various stresses (VIRGILIO et al. 1994; Guo and Olsson 2014). The trehalose biosynthetic genes are regulated by stress induced transcription factors Msn2 and Msn4 (Gasch et al. 2000). As such, trehalose accumulation is a known indicator of stress response in *S. cerevisiae*.

Nutrient addition during bioethanol production has been reported to be costly (Jones and Ingledew 1994; Pereira et al. 2010b). Therefore, it is important to identify which nutrients are required when propagating *S. cerevisiae*. Substrate used in propagation of *S. cerevisiae* needs an elemental composition that is sufficient to support cell growth in order to allow for cultivation without addition of other nutrients. When comparing the elemental composition of *S. cerevisiae* cell mass with that of molasses (Table 3.1.1), it is revealed which inorganic nutrients need to be supplied to *S. cerevisiae* propagation. Beet molasses appears deficient in nitrogen and magnesium, whereas cane molasses lacks nitrogen only.

Table 3.1.1 Comparison of baker's yeast, beet and cane molasses elemental compositions.

	C (g)	N (g)	P (g)	K (g)	Mg (g)	Ca (g)
100 g compressed yeast (27% solids) (Reed and Nagodawithana 1991)	12.3	2.30	0.28	0.54	0.03	0.01
100 g beet molasses (Reed and Nagodawithana 1991)	33.0	1.50	0.03	6.00	0.025	0.30
100 g cane molasses (Palmonari et al. 2020) ^a	62.3	0.46 ^b	2.03 ^c	1.82	0.43	1.39
100 g beet molasses (Palmonari et al. 2020) ^a	62.1	0.06 ^b	0.76 ^c	2.44	0.02	0.30

^a Values are averages of 16 samples of molasses.

^b Nitrites

^c Phosphates

3.3 Substrate – Lignocellulosic hydrolysate

Lignocellulosic biomass, found in plants and trees, is predominantly composed of cellulose, hemicellulose and lignin (Palmqvist and Hahn-Hägerdal 2000). Its components are tightly linked together to form a recalcitrant structure that gives rigidity to the plants or trees it is a part of. The cellulose and hemicellulose generally make up about two thirds of the lignocellulosic biomass and because they are carbohydrates, they form the fraction of lignocellulosic biomass that can be utilized for bioethanol production (Sun and Cheng 2002; Almeida et al. 2007). Cellulose consists of glucose units in a uniform structure, whereas

hemicellulose can be very heterogenous. Hemicelluloses can contain pentoses, hexoses and/or uronic acids and small amounts of other sugars such as rhamnose and fucose (Gírio et al. 2010). Xylans are the most abundant hemicelluloses as they are the main components of secondary cell walls in plants. They constitute 20-30% of the biomass of hardwoods and herbaceous plants and in some grasses and cereals xylans can account for up to 50% (Ebringerová et al. 2005).

For optimal bioethanol yields it is important to convert xylose to ethanol as xylans are so abundant in plant biomass. However, *S. cerevisiae* naturally does not have the capability to convert xylose. To mitigate this, studies have introduced xylose converting genes into *S. cerevisiae* as reviewed by Hahn-Hägerdal et al. (2001); Kim et al. (2013) and Lee et al. (2021).

One approach relies on inserting xylose reductase (Xyl1) and xylitol dehydrogenase (Xyl2) genes from *Pichia stipitis*; another one is based on the introduction of xylose isomerase (XylA) from *Piromyces*. Both strategies yield xylulose and feed into the non-oxidative branch of the pentose phosphate pathway via xylulose 5-phosphate, which is formed by xylulokinase.

The recalcitrant structure of lignocellulosic biomass needs to be broken open for hydrolytic enzymes to gain access to cellulose fibers. Therefore, in bioethanol production, pretreatment of lignocellulosic biomass generally starts with the solubilization of hemicellulose by impregnation with acid, water, steam, organic solvents or alkaline agents. The most commonly used methods are acid, water, steam or a combination of the three (Gírio et al. 2010). The impregnation, followed by incubation at elevated temperatures, produces liquids that are rich in monomeric or oligomeric sugars from the hemicellulose fraction and solids that are rich in cellulose which can be treated with hydrolytic enzymes. The solid fraction is subsequently enzymatically hydrolyzed to obtain a hexose rich liquid fraction. The pentose and hexose liquid hydrolysate fractions can be separately fermented by *S. cerevisiae* or combined. Combining the fractions is better for cost-effectiveness from a capital cost point-of-view, however, fast co-consumption of pentose and hexose sugars in *S. cerevisiae* is a challenge due to glucose repression (See: **Chapter 2.2**; Subtil and Boles 2012).

Not only sugars are produced during the pretreatment process. Degradation products (i.e., LCIs) are formed from the sugars (furans and weak acids) and from lignin (phenolics) (Figure 3.3.1.; Olsson and Hahn-Hägerdal 1996). LCIs that are commonly found in wheat straw and corn stover hydrolysates are formic acid, acetic acid, 5-hydroxymethyl furfural (HMF) and furfural.

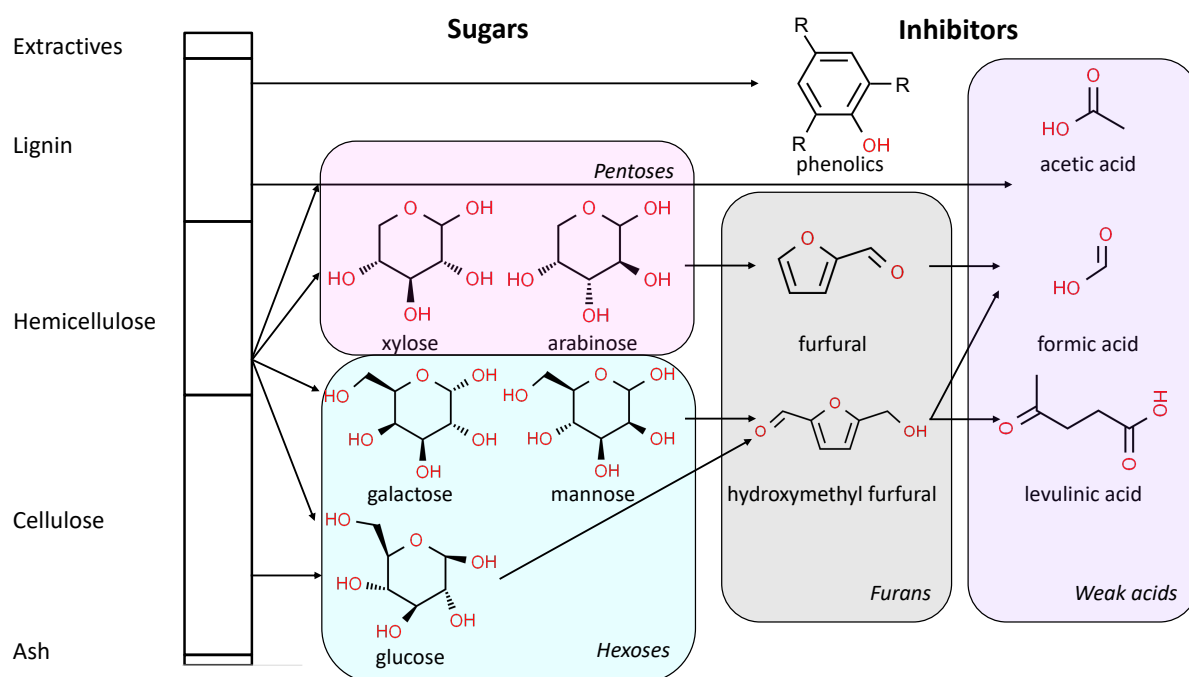


Figure 3.3.1. Degradation products formed during pretreatment of lignocellulosic biomass which act as inhibitors during the bioethanol fermentation process. Figure adapted from Palmqvist and Hahn-Hägerdal (2000) and Almeida et al. (2007).

As described previously, batch-to-batch variability can result in a different LCI profile even when using the same pretreatment method and the same type of raw biomass. Table 3.3.1 shows the heterogeneity of different batches of wheat straw hydrolysate (WSH) used in this thesis. However, a general trend in inhibitor profiles can be observed. Typically, when lignocellulosic biomass is incubated for longer or at higher temperatures, degradation of the sugars and intermediate furans results in more weak acids than furans.

Table 3.3.1. Composition of different lignocellulosic hydrolysates used in the papers in this thesis. The different wheat straw hydrolysates were produced using the same method but show a difference in inhibitor profile.

	Glucose ^b	Xylose ^b	Formic acid ^b	Acetic acid ^b	HMF ^b	Furfural ^b
WSH ^a (paper I , development screen)	68.8	36.4	1.2	4.7	0.6	3.0
WSH ^a (paper I , validation screen)	80.0	30.6	0.8	7.7	0.3	4.2
WSH ^a (paper II)	75.3	38.2	1.6	5.1	0.1	2.8
WSH ^a (paper III)	68.8	36.4	1.2	4.7	0.6	3.0
WSH ^a (paper IV)	64.9	23.8	1.0	5.1	0.3	2.2
WSH (Large scale study) ^c	64.9	27	0.8	5.3	1.2	3.2

^a Wheat straw hydrolysate

^b Concentrations are given in g L⁻¹

^c Study involving large scale propagation

4. Stress effects and response in yeast

Stress is known to affect *S. cerevisiae* metabolism in a variety of ways. Although it was long believed that industrial propagation conditions were optimal for *S. cerevisiae* growth, studies have reported that cells do encounter stress under industrial conditions resulting in reduced biomass yield, fermentative capacity, and cell viability (Attfield 1997; Pérez-Torrado et al. 2005). In addition to such stresses, LCIs, formed during pretreatment of lignocellulosic biomass, are known to inhibit *S. cerevisiae* metabolism in hydrolysate-containing fermentations. The effect of LCIs on *S. cerevisiae* metabolism has been studied extensively and has been reviewed by Piotrowski et al. (2014), Cunha et al. (2019), Brandt et al. (2019) and others. This chapter will describe stress experienced by *S. cerevisiae* during industrial propagation as well as in hydrolysate-containing medium and the effects of these stresses on metabolism. Additionally, the response mechanisms of *S. cerevisiae* to the encountered stresses will be described. An overview of described inhibitory effects of LCIs on *S. cerevisiae* metabolism and the cellular responses to these effects can be found in Table 4.1 and Figure 4.1.

4.1 Intracellular acidification & ATP depletion

Weak acids are often present in hydrolysates (**Figure 3.3.1; Table 3.3.1**) and are thought to be one of the key components to the inhibitory nature of hydrolysates for yeast growth (Guo and Olsson 2014). The weak acids in the hydrolysate diffuse freely over the cell membrane when extracellular pH is lower than the dissociation constant. Operating at low pH is generally desirable in industry because it removes the need for pH control, which can be costly, and it reduces the risk of contamination. The acids enter the cell in their protonated form and dissociate in the cytoplasm, creating protons and thereby acidifying the intracellular pH (Ullah et al. 2012). The cellular response of *S. cerevisiae* to intracellular acidification is to export protons at the expense of ATP using proton-translocating ATPases (Eraso and Gancedo 1987; Chambel et al. 1999). The anionic forms of the acids are

presumed to be exported by multidrug resistance (MDR) transporters which also require ATP (Schuller et al. 2004; Ullah et al. 2012). Several important enzymes in glycolysis and the ethanol fermentation pathways have been shown to be inhibited by the presence of weak acids and furans (Banerjee et al. 1981; Pampulha and Loureiro-Dias 1990; Modig et al. 2002), thereby inhibiting the regeneration of ATP.

4.2 Oxidative stress

Reactive oxygen species (ROS) are formed as a result of the presence of different LCIs in the cytosol causing oxidative damage. Weak acids cause ROS formation due to increased proton levels in the cytosol. Furans, such as furfural and HMF, are known to function as thiol-reactive electrophiles thereby generating ROS (Allen et al. 2010; Kim and Hahn 2013). Phenolic compound vanillin has been reported to induce ROS formation (Nguyen et al. 2014a) although the mechanism for this is not yet elucidated. If ROS accumulate in the cell, they cause damage to DNA, proteins, lipids and the cytoskeleton (Attfield 1997; Rowe et al. 2008; Allen et al. 2010).

The primary strategy of *S. cerevisiae* in response to oxidative stress is to synthesize antioxidants that either scavenge ROS such as glutathione and thioredoxin (Stephen et al. 1995) or to synthesize metallothioneins and superoxide dismutases (Tamai et al. 1993) and catalases (Grant et al. 1998) that detoxify ROS. *S. cerevisiae*'s secondary strategy to oxidative stress involves DNA repair, tagging oxidized proteins for degradation, and reduction of oxidized glutathione and thioredoxin as reviewed by Jamieson (1998). The reduction of oxidized glutathione and thioredoxin requires NADPH and as such leads to upregulation of pathways that supply NADPH such as the pentose phosphate pathway. Martin et al. (2008) found that 100 h after inoculation of wine yeast propagation, catalase levels had increased to 50 units/g protein, indicating the presence of oxidative stress. Another study on wine yeast propagation showed a 2-fold increase in expression of Mn superoxide dismutase Sod2 towards the end of propagation (Gómez-Pastor et al. 2010a).

During fed-batch propagation of yeast, oxidative stress is present throughout most of the process. As discussed in **Chapter 2**, propagation cultures are aerated and the respiratory metabolism of *S. cerevisiae* creates ROS (Attfield 1997). A study on the propagation process found that towards the end of the feed phase, gene expression levels return to values similar to those of the mid-exponential growth phase during the batch with the exception of genes relating to oxidative stress response, the electron transport chain and protein transport (FMP genes) which all remain upregulated (Gómez-Pastor et al. 2010a). Certain glycolytic proteins (Tdh3p, Pdc1p, Ad1p and Eno1p) have been found to be specifically oxidized in the presence of ROS (Le Moan et al. 2006), which has been hypothesized to lead to loss of glycolytic flux (Gómez-Pastor et al. 2010a). The overexpression of TRX1 and TRX2, encoding thioredoxins, has been shown to improve cell mass yields during propagation, further supporting this hypothesis (Gómez-Pastor et al. 2010b; Unrean et al. 2018). Proteomic data showed an increase of certain mitochondrial proteins (Cor1p, Pst2p, Sod2p and Rip1p)

towards the end of the fed-batch indicating an increased demand for mitochondrial biogenesis. Additionally, FMP genes were found upregulated that are involved in cytosol-to-mitochondria transport of enzymes such as catalase, superoxide dismutase and cytochrome c oxidase further supporting the importance of the mitochondria in this phase (Gómez-Pastor et al. 2010a).

4.3 Redox imbalance

In *S. cerevisiae*, toxicity of certain furan and phenol aldehydes is mitigated by detoxification to less toxic corresponding acids under aerobic conditions (Sárvári Horváth et al. 2003) and alcohols under anaerobic conditions (Ask et al. 2013a). NAD(P)H is involved in these reactions and can lead to a redox imbalance, unless these redox factors can be regenerated (Ask et al. 2013; Nguyen et al. 2014b). Furan and phenol detoxification specifically interferes with the oxidative stress response because NADPH is required for regeneration of glutathione and therefore competes for the redox factors. Thioredoxins encoded by *TRX1/2* are capable of regenerating NADPH and they have been suggested to play a role in redox balancing under LCI stress (Unrean et al. 2018). Although the pentose phosphate pathway is a major source of NADPH supply, NADPH can also be supplied by acetaldehyde dehydrogenase, encoded by *ALD5/6*, through conversion of acetaldehyde to acetate (Saint-Prix et al. 2004) or through the NADP⁺ specific isocitrate dehydrogenase catalyzing oxidation of isocitrate to α -ketoglutarate (Haselbeck and McAlister-Henn 1991). If the redox imbalance requires increased NADH supply, NAD⁺ specific aldehyde dehydrogenases, encoded by *ALD2/3/4*, are able to regenerate NADH (Navarro-Aviño et al. 1999). However, expression of these genes is glucose repressed. NADH regeneration is also possible via glycolysis through glyceraldehyde 3-phosphate encoded by *TDH1/2/3* (McAlister and Holland 1985) or the TCA cycle through NAD⁺ dependent isocitrate dehydrogenase encoded by *IDH1/2* (Cupp and McAlister-Henn 1992), malate dehydrogenase encoded by *MDH1* (McAlister-Henn and Thompson 1987) or malic enzyme encoded by *MAE1* (Boles et al. 1998).

Under anaerobic conditions, biosynthesis in *S. cerevisiae* leads to excess NADH production (van Dijken and Scheffers 1986; Verduyn et al. 1990). In order to close the redox balance for NAD⁺/NADH glycerol is formed during anaerobic growth (Gancedo et al. 1968; Oura 1977). Reduction of furfural has been reported to replace glycerol formation for the regeneration of NAD⁺ under anaerobic conditions (Wahlbom and Hahn-Hägerdal 2002). However, limits to the oxidative capacity constrain the extent to which this can occur (Sárvári Horváth et al. 2003; Pornkamol and Franzen 2015).

In addition to redox imbalance due to encountered LCI stress, the introduced xylose conversion genes may contribute to a redox imbalance in the strains used in **papers I – IV**. The strains used in these papers had the xylose reductase (Xyl1) and xylitol dehydrogenase (Xyl2) genes from *Pichia stipitis* introduced into their genome. The xylose reductase that is

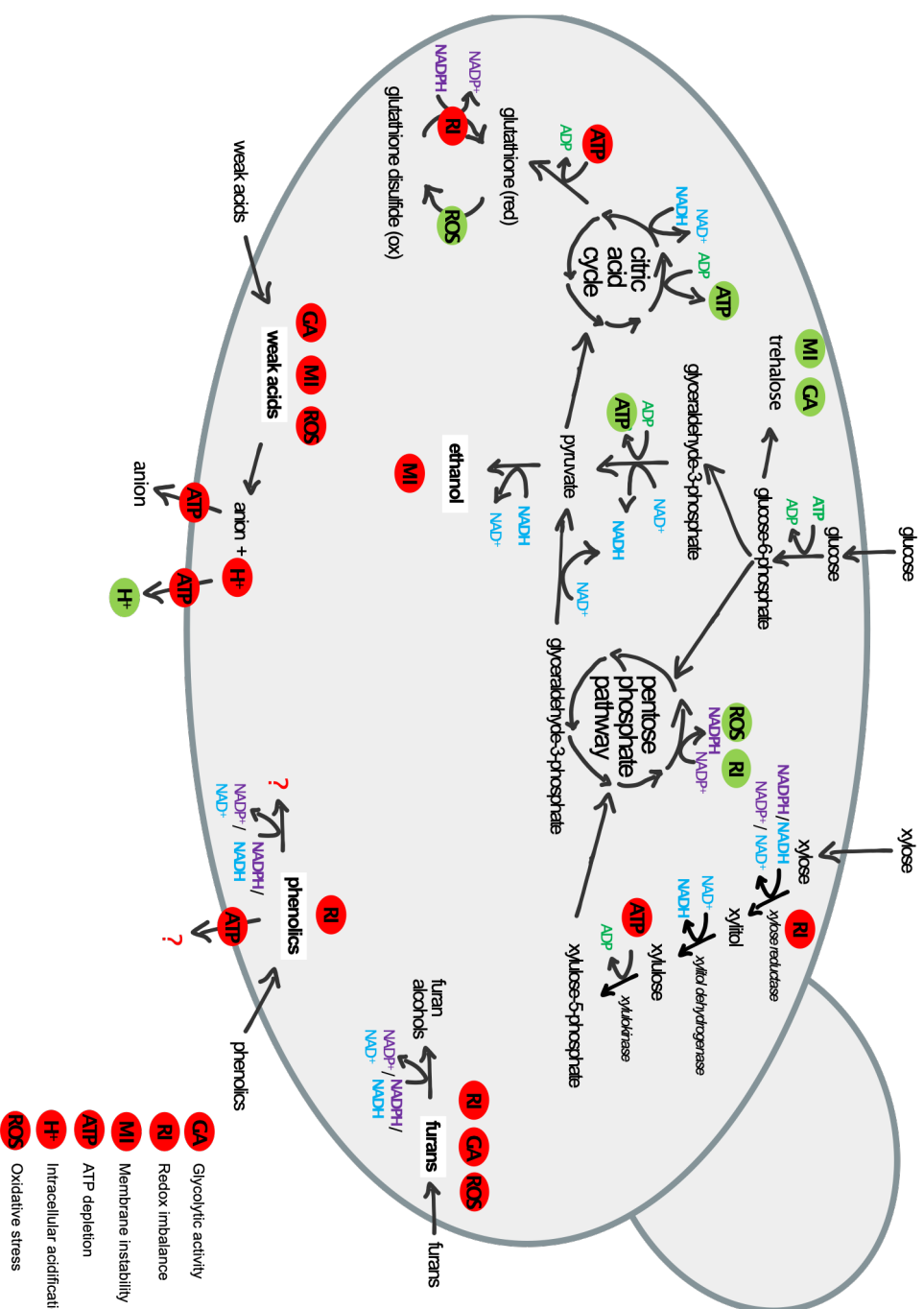


Figure 4.1. A schematic overview of the effects of lignocellulosic inhibitor stress on *S. cerevisiae* and corresponding stress response on cellular resources ATP, NADH and NADPH. Red icons indicate effects that inhibitors have on physiology and green icons indicate stress response mechanisms. Main inhibitors (weak acids, furans, phenolics and ethanol) are indicated by a white box. NAD⁺/NADH is indicated in blue. NADP⁺/NADPH is indicated in purple. ADP/ATP is indicated in green when the effect is positive and in red when the effect is negative. Question marks indicate suggested mechanisms.

introduced uses both NADPH and NADH as a cofactor while the xylitol dehydrogenase is strictly NAD⁺ dependent (Eliasson et al. 2000). This discrepancy in cofactor specificity has been reported to lead to xylitol accumulation in the absence of respiration (Kötter and Ciriacy 1993).

4.4 Structural damage

Several LCIs can inflict damage on structural components of the cell. Weak acids have been reported to cause decreased ergosterol content in the membrane leading to membrane instability and increased permeability (Godinho et al. 2018). Compromised permeability of the cell membrane leads to influx of other LCIs and therefore is considered to be a contributor to the synergistic toxicity of lignocellulosic hydrolysates (Ding et al. 2011).

Trehalose is thought to confer stress protection to a variety of stresses such as weak acids (Guo and Olsson 2014) and high temperatures (Mensonides et al. 2014) by preventing denaturation and aggregation of proteins (Singer and Lindquist 1998) and by stabilizing the cell membrane (Mansure et al. 1994; Cray et al. 2015). Trehalose has also been found upregulated in response to ethanol stress, which is relevant as ethanol accumulates at the end of the batch phase (Alexandre et al. 2001).

4.5 Osmotic stress

At the start of the batch phase sugar concentrations in the medium are high, causing osmotic stress (Pérez-Torrado et al. 2005). This can lead to loss of turgor due to diffusion of water out of the cell. Additionally, hyperosmolarity can lead to growth inhibition and loss of fermentative capacity. A well-documented response to osmotic stress is the high-osmolarity glycerol response pathway (Brewster et al. 1993), which induces the genes *GPD1* and *GPP2* involved in glycerol synthesis. The production of intracellular glycerol has been shown to prevent efflux of water from the cells (Hohmann 2002). Glycerol biosynthesis requires the input of NADH and therefore can cause redox imbalance under hyperosmotic stress conditions.

In summary, the mechanisms involved in mitigating LCI stress require cellular resources such as ATP, NADH, and NADPH (Figure 4.1). ATP is required to cope with the efflux of inhibitors and to maintain intracellular pH by the efflux of protons. NADH and NADPH have been shown to be required for detoxification of, for instance, aldehydes such as furfural (Miller et al. 2009; Jarboe 2011). Cellular repair mechanisms, necessary to repair damage to macromolecules and membranes as a result of LCIs, require a wide range of resources including ATP, NADPH, carbon, sulfur, and nitrogen, as reviewed by Piotrowski et al. (2014). Thus, these stress response mechanisms compete for a limited amount resources and as such the extent to which the different stress responses are activated needs to be delicately balanced.

Table 4.1. Overview of described inhibitory effects of LCIs on *S. cerevisiae* metabolism and the cellular responses to these effects.

Inhibitory effect	Compounds that contribute to effect	Described reason for effect	Described cellular response	References
Intracellular acidification & ATP depletion	Weak acids ^a Phenolics ^b	Diffusion of acids into the cell and subsequent increase of H ⁺ in the cell. Export occurs via MDR transporters or H ⁺ -ATPases which require ATP.	Weak acids lead to activation of Msn2/4 and thereby trehalose accumulation which stabilize the cell membrane. Phenolics lead to detoxification. Respiratory metabolism is required to mitigate ATP depletion. Production of antioxidants to scavenge or detoxify ROS. Pentose phosphate pathway is upregulated to increase NADPH supply needed to replenish antioxidants. Pentose phosphate pathway and TCA cycle is upregulated to increase NAD(P)H supply. Respiratory metabolism to feed lipid/sterol metabolism.	(Bellissimi et al. 2009; Ullah et al. 2012; Palma et al. 2018)
Oxidative stress	Weak acids ^a Phenolics ^b	Oxidation of cellular macromolecules, mitochondria and membrane by ROS.		(Rowe et al. 2008; Allen et al. 2010; Kim and Hahn 2013; Nguyen et al. 2014a; Guo and Olsson 2014)
Redox imbalance	Phenolics ^b Furans ^c	Detoxification of phenolics and furans consume NAD(P)H.		(Ask et al. 2013a; Nguyen et al. 2014b; Adeboye et al. 2017)
Cell wall and membrane instability	Weak acids ^a	Increased permeability of the cell wall and membrane causes leaking of vital compounds out of the cell as well as increased diffusion of inhibitors into the cell.		(Ding et al. 2011; Godinho et al. 2018)
Inhibition of enzymes	Weak acids ^a Furans ^c	Hexokinase, Glyceraldehyde-3-phosphate dehydrogenase and alcohol dehydrogenase are most strongly inhibited by furfural.		(Banerjee et al. 1981; Pampulha and Loureiro-Dias 1990; Modig et al. 2002)

^a Acetic acid, formic acid, levulinic acid

^b Ferulic acid, coumaric acid, vanillin, syringaldehyde, coniferyl alcohol, eugenol, acetovanillin, feruloyl amide, coumaryl amide and others

^c Furfural, 5-hydroxymethyl furfural, 2-furoic acid

5. Strategies to combat inhibition of yeast metabolism

Industrial *S. cerevisiae* fermentation requires large amounts of cells for inoculation because the lack of molecular oxygen in the medium prevents cell growth beyond a few doublings. As cell titers are directly correlated to volumetric ethanol productivity it is critical to inoculate high cell titers (Cahill et al. 2000; Stewart 2017). Additionally, the inhibitory nature of lignocellulosic hydrolysate, which is abundantly present in fermentation medium for second generation bioethanol production, contributes to loss of cell viability and therefore to further loss of volumetric ethanol productivity. Therefore, it is crucial in second generation bioethanol processes to supply an inoculum that is both large enough in size as well as in a robust metabolic state to prevent loss of viability. This chapter explains why short-term adaptation of *S. cerevisiae* to LCIs is the main subject investigated in this thesis. First, I introduce alternate methodologies to combat the loss of *S. cerevisiae* viability during hydrolysate fermentation. Then, I explain why short-term adaptation to LCIs merits further investigation and discuss findings from the papers contained in this thesis.

5.1 Detoxification

Detoxification of the hydrolysate prior to fermentation has been investigated as a strategy to improve fermentability in numerous of studies. Examples of methods that have been investigated include alkali treatment (Van Zyl et al. 1988), evaporation (Palmqvist et al. 1996), anion exchange and laccase (phenoloxidase) treatment (Jönsson et al. 1998), activated carbon (Duque et al. 2015; Kim et al. 2016), lignin blocking agents (Yang and Wyman 2006) and detoxification by microorganisms such as *Trichoderma reesei* (Palmqvist et al. 1997) or *Acinetobacter baylyi* (Kannisto et al. 2015). These studies showed varying levels of success in removing inhibitors from the hydrolysate and thereby increasing the fermentability. However, the detoxification methodologies have limitations in their usefulness in industry.

Chemical treatments generally require separate operational steps in the bioethanol production process, which makes them economically unfavorable. As an example, a simple detoxification step using calcium hydroxide and heating to 95 °C was estimated to constitute 22% of the total ethanol production cost (von Sivers et al. 1994). Detoxification by microorganisms has been shown to lead to significant loss of fermentable sugars (35%) (Larsson et al. 1999). Treatment with enzymes could theoretically be incorporated into an existing operational step in the production process which is an advantage, however enzyme cost would nevertheless be inhibitory (Kudanga and Le Roes-Hill 2014).

5.2 Strain engineering

Another strategy to cope with the inhibitory nature of lignocellulosic hydrolysates is to improve robustness of *S. cerevisiae* to the LCIs. A wide range of studies has focused on rational engineering, overexpressing individual genes or sets of genes, to confer tolerance to individual inhibitors present in lignocellulosic hydrolysates. In one example, the co-overexpression of transaldolase (*TALI*) and alcohol dehydrogenase (*ADHI*) encoding genes has been shown to improve final ethanol titers in the presence of 70 mM furfural by 2.3-fold compared to the parental strain (Hasunuma et al. 2014). In another study, the overexpression of *ALD5*, *PADI*, *ATF1* and *ATF2* lead to fast conversion of the phenolic compounds coniferyl aldehyde and ferulic acid (Adeboye et al. 2017). The volumetric conversion rate of coniferyl aldehyde improved by 13% and of ferulic acid improved by 30% compared to the control. In a final example, the co-overexpression of *TPS1* and *ARI1* combined with deletion of *NTH1* resulted in a strain that exhibited tolerance to ethanol up to 14% whereas the parental strain was inhibited at 6% ethanol (Divatte et al. 2017). This engineered strain showed improved tolerance to a mixture of 30 mM furfural and 30 mM HMF compared to the parental strain.

Rational metabolic engineering has also been successfully applied to confer tolerance to more general stress elements. Deleting the acetate transporter gene *ADY2* improved growth under acetic acid, ethanol and hydrogen peroxide stress. It also led to a 1.6-fold increase in volumetric ethanol productivity of industrial strain 4126 in the presence of 7.5 g L⁻¹ acetate at pH 4.5 compared to its parental strain (Zhang et al. 2017). Increased NADP⁺/NADPH cycling has been shown to be a successful strategy to combat furfural stress. Overexpression of *ZWF1*, encoding the NADP⁺ dependent glucose-6-phosphate dehydrogenase, has been shown to allow growth of *S. cerevisiae* at furfural concentrations that are toxic to the parental strain (Gorsich et al. 2006; Cunha et al. 2015). Overexpression of genes that stimulate glutathione production has been shown to improve robustness to spruce hydrolysate by improving oxidative stress tolerance (Ask et al. 2013b; Kim and Hahn 2013). Kim and Hahn (2013) showed that constitutively expressing transcription factor Yap1, as well as catalases Cta1 and Ctt1 improved growth in the presence of furfural and HMF, whereas overexpression of glutathione biosynthetic genes improved growth in the presence of furfural but not HMF. Ask et al. (2013b) showed that a strain overexpressing *GSH1* and *GLR1*, encoding enzymes involved in glutathione metabolism, improved the ethanol yield

on hexoses by 1.7-fold in simultaneous saccharification and fermentation of pretreated spruce.

Table 5.2.1. Examples of adaptive laboratory evolution of *S. cerevisiae* strains for improved performance in lignocellulosic hydrolysate fermentation.

Strain name	Selective pressure	Change in performance ^a	Reference
TMB3400	Xylose consumption and fast growth	μ_{\max}^b : 3.5-fold; $Y_{e/s}^c$: 1.5-fold; q_e^d : 1.8-fold	Wahlbom et al (2003)
TMB3400-FT30-3	Furan tolerance	Decreased lag phase by 80%	Heer et al. (2009)
<i>S. cerevisiae</i> F12	Ethanol production and wheat straw hydrolysate tolerance	Q_e^e : 2-fold; C_e^f : 1.8-fold	Tomás-Pejó et al. (2010)
TMB3720	Spent sulfite liquor tolerance	$Y_{e/s}^c$: 1.8-fold q_e^d : 4-fold;	Sánchez i Nogué et al. (2012)
KE1-17	Spruce hydrolysate tolerance	q_e^d : 1.3-fold; q_t^g : 1.6-fold	Koppram et al. (2012)
Ethanol Red isolate ISO12	Spruce hydrolysate & heat tolerance	$Y_{e/s}^c$: 7.6-fold	Wallace-Salinas and Gorwa-Grauslund (2013)
CEN.PK 113–7D isolate BH13	Coniferyl aldehyde tolerance	Decreased lag phase by 75%; cross-resistance for vanillin and ferulic acid	Haclsalihoglu et al. (2019)
SC90	Casava pulp hydrolysate tolerance	Decreased lag phase by 57%; Q_e^e : 1.5-fold	Palakawong Na Ayutthaya et al. (2019)

^a Changes in performance are expressed as a fold-change compared to the non-evolved parental strain under the same conditions.

^b maximum specific growth rate

^c ethanol yield on total fermentable sugars

^d specific ethanol productivity

^e volumetric ethanol productivity

^f final ethanol concentration

^g specific furfural detoxification rate

A clear disadvantage to rational engineering strategies is that using genetically modified strains in industry requires an increased number of regulations to be complied with and thereby increases costs. Evolutionary engineering approaches do not face such difficulties and as such show good potential to develop industrial strains with improved performance in lignocellulosic hydrolysates. An overview of the numerous examples that can be found in literature is shown in Table 5.2.1.

Strain engineering methods are continuously advanced and the development has led to the ability to construct strains at an increasing rate (Petzold et al. 2015). Screening of strains in

hydrolysate medium at high throughput is difficult due to the incompatibility of the conventional absorption-based method for determining of cell density. The Biolector platform is a multi-well cultivation system that uses scattered light instead of absorption to measure cell density and thus it was tested for compatibility with dark hydrolysate medium.

Aerobic growth of industrial strain KE6-12 in wheat straw hydrolysate using the Biolector system showed good reproducibility ($n = 3$; **Paper I, Figure 1**). The obtained growth profiles showed a clear distinction between cultures that had been inoculated at different OD-values (**Paper I; Figure 1A and 1C**) and between cultures that had been subjected to short-term adaptation and non-adapted cultures (**Paper I: Figure 1C**). Similarly, good reproducibility was obtained for cultures grown anaerobically (**Paper I: Figure 1D**), using a different hydrolysate (corn stover; **Paper I: Figure 1E**) or a different strain (CR01; **Paper I: Figure 1E**). Ultimately, the developed cultivation method was applied in a screening of different *S. cerevisiae* strain backgrounds in which *PDR12* had been deleted. Improved aerobic growth was observed for the CEN.PK113-7D $\Delta Pdr12$ strain compared to the parental strain, but not for many of the other strains that were screened (**Paper I, Table 1**). Therefore, it was demonstrated that the developed method allows for high-throughput investigation of how strain background affects the result of a single gene modification in dark hydrolysates. Increasing the throughput capacity of hydrolysate cultivation systems, as resulted from the work in **Paper I**, will allow for a more systematical investigation of, for example, clone libraries or different strain backgrounds in dark hydrolysate medium. Systematic investigation of different hydrolysates compositions on growth could also be facilitated using such high throughput cultivation methods.

A disadvantage of both rational and evolutionary metabolic engineering strategies is that they optimize *S. cerevisiae* performance towards a specific LCI profile. As described in **Chapter 4** of this thesis, LCI profiles in hydrolysates are subject to high variability depending on the raw biomass used (Klinke et al. 2004; Almeida et al. 2007), differences in seasonal weather (Bunnell et al. 2013; Greenhalf et al. 2013), and pretreatment methods used (Chundawat et al. 2010). Strains that are optimized through metabolic engineering risk performing optimal in a specific hydrolysate, but sub-optimal in others.

5.3 Nutrient supplementation

The addition of nutrients to lignocellulosic fermentation medium has been shown to improve fermentation performance (Jørgensen 2009; Xiros and Olsson 2014; Kelbert et al. 2015). Manganese supplementation has been reported to improve xylose consumption under acetic acid stress (Ko et al. 2016), while the addition of complex nutrients to high-gravity lignocellulose fermentation has been shown to improve the fermentation performance (Xiros and Olsson 2014). Supplementation of the fermentation medium with nutrients, even in non-hydrolysate media, is a well-known strategy that can improve ethanol tolerance and thereby ethanol productivity (Dombek and Ingram 1986; Winter et al. 1989; Alfenore et al. 2002; Zhao et al. 2009). The improved performance observed in these works was attributed to

extended exponential growth, improved viability, or a shift in metabolism from glycerol to ethanol formation.

In research on the fermentation of lignocellulosic hydrolysates the nutrient composition of the minimal medium, as optimized by Verduyn et al. (1992), or a complex nutrient source such as yeast extract, is often used (Table 5.3.1). However, these nutrient sources are not industrially relevant due to their high cost, and inexpensive nutrient sources such as corn steep liquor or urea are used instead (Jones and Ingledew 1994; Pereira et al. 2010b). The effectiveness of nutrient addition appears hydrolysate or strain dependent as the addition of biotin and ammonium to wheat straw hydrolysate led to an increase in $Y_{e/s}$ of 22%, whereas the same addition to spruce led to a 39% increase (Table 5.3.1).

Due to large fermentation volumes, it might be a more economically attractive strategy to supplement the propagation with nutrients that improve robustness provided that the robust phenotype remains active during the subsequent fermentation. In order to investigate this idea, nutrients, known to influence LCI tolerance in *S. cerevisiae*, were added to the propagation medium before inoculating the propagated cells in hydrolysate-containing fermentation medium (**Paper II**). Screenings were performed using the Biolector method as described in **paper I**. When a mixture of pyridoxine, thiamine and biotin was added to the propagation, ethanol yields during subsequent fermentation improved from 0.04 g g⁻¹ to 0.19 g g⁻¹ in wheat straw and from 0.02 g g⁻¹ to 0.08 g g⁻¹ in corn stover hydrolysate (**Paper II, Table 5**). Other nutrient additions that were tested but showed no improved fermentation are zinc sulfate, manganese chloride, iron sulfate as well as different nitrogen sources urea, ammonium sulfate and peptone.

Short-term adaptation during propagation, without any nutrient addition, resulted in ethanol yields of 0.37 g g⁻¹ in wheat straw hydrolysate fermentation and 0.33 g g⁻¹ in corn stover hydrolysate fermentation. Combining the addition of the mixture of nutrients with the short-term adaptation improved ethanol yields 0.43 g g⁻¹ in wheat straw hydrolysate fermentation and 0.41 g g⁻¹ in corn stover hydrolysate fermentation. These results are further emphasized by improved growth that was observed during these fermentations (Figure 5.3.1). Interestingly, addition of the vitamin mixture improved growth in corn stover hydrolysate to similar levels as short-term adaptation does (Figure 5.3.1B) while this is not the case for wheat straw hydrolysate (Figure 5.3.1A). This indicates that the different hydrolysates are lacking different nutrients required for growth of *S. cerevisiae*.

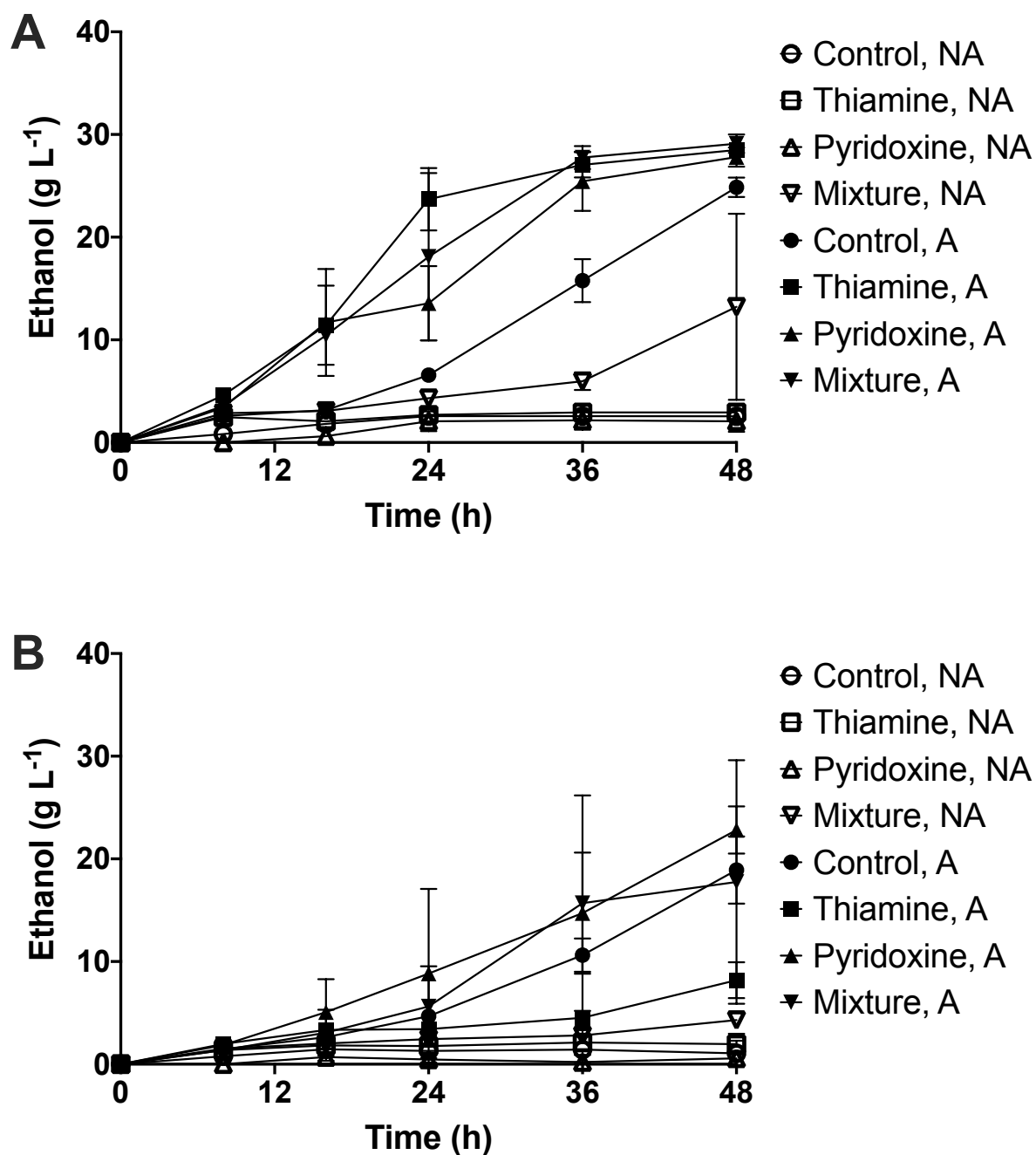


Figure 5.3.1. Influence of nutrient addition to propagation of *S. cerevisiae* on growth during subsequent fermentation of (A) wheat straw hydrolysate and (B) corn stover hydrolysate. Nutrient addition to the propagation was investigated in combination with short-term adaptation to the hydrolysate (closed symbols, 'A') and without short-term adaptation (open symbols, 'NA'). The mixture of nutrients consisted of biotin, thiamine and pyridoxine.

5.4 Short-term adaptation

When producing an inoculum that is suitable for lignocellulose fermentation it is not sufficient to maximize cell mass yield while keeping process times as short as possible. Another important aim of propagation is to produce highly robust cells that are protected against stress during harvesting, storage and the lag-phase during the subsequent

fermentation. This can be achieved by stopping the feed at the end of propagation, while continuing to aerate (Oura 1974), a practice that is implemented in baker's yeast production. During this time, unused nutrients are taken up by the cells. Budding cells divide but non-budding cells do not initiate budding causing the cell population to synchronize in the cell cycle. The relief from carbon- and nitrogen-repression causes the activation of the gluconeogenic pathway and results in accumulation of trehalose (Rose and Vijayalakshmi 1993). In addition to trehalose accumulation, the carbon and nitrogen starvation phase is thought to induce a wider stress response through activation of heat shock transcriptional regulators conferring cross-protection against several stresses (Hahn and Thiele 2004; Gómez-Pastor et al. 2011).

Another way in which process conditions can be used to improve robustness of the produced inoculum is short-term adaptation. Hydrolysate supplementation to the propagation medium in non-lethal concentrations has been shown to improve *S. cerevisiae* tolerance to LCIs resulting in higher viability, biomass production, and fermentation capacity during subsequent fermentation in the same hydrolysate (Table 5.4.1; **Paper III**). Nielsen et al. (2015) have also shown that short-term adaptation improves performance in simultaneous saccharification and co-fermentation. The cellular mechanisms underlying short-term adaptation are, however, still poorly understood.

In order to investigate the effect of strain background on the effect of short-term adaptation to hydrolysates, physiological parameters of two closely related *S. cerevisiae* strains, KE6-12 and CR01, were evaluated in fed-batch cultivation in bioreactors (**Paper III**). Both strains were subjected to short-term adaptation during the feed phase. As a result of short-term adaptation, specific ethanol productivity of KE6-12 and CR01 during subsequent hydrolysate fermentation was found to be increased by 1.6-fold and 3.6-fold, respectively (Table 5.4.1).

Physiological parameters that are thought to be indicators of stress robustness (i.e., intracellular trehalose and glycogen content and cell viability) were evaluated after 37 h of feeding. Glycogen accumulation was not altered upon short-term adaptation of strain KE6-12 (**Paper III, Figure 3B**). However, although CR01 accumulated $19.6 \text{ g}_{\text{glycogen}} \text{ g}_{\text{CDW}}^{-1}$ when grown in the control medium, no glycogen accumulation was observed upon short-term adaptation (**Paper III, Figure 3A**). This result, combined with the observed ethanol accumulation during the feed phase, indicates that CR01 might experience glucose repression despite the low feed rate that was used ($\mu = 0.05^{-1}$). Trehalose accumulation for strain KE6-12 increased from $28.9 \text{ g}_{\text{trehalose}} \text{ g}_{\text{CDW}}^{-1}$ during the control cultivation to $50.7 \text{ g}_{\text{trehalose}} \text{ g}_{\text{CDW}}^{-1}$ during short-term adaptation (**Paper III, Figure 3D**). As described in **Chapter 4.4**, trehalose content of cells is thought confer stress protection by preventing denaturation and aggregation of proteins (Singer and Lindquist 1998) and by stabilizing the cell membrane (Mansure et al. 1994; Cray et al. 2015). Intracellular trehalose accumulation is often reported in conjunction with stressful growth conditions (REF) and as such, is considered to be a general stress indicator. However, trehalose accumulation for strain CR01 decreased from $38.8 \text{ g}_{\text{trehalose}} \text{ g}_{\text{CDW}}^{-1}$ during the control cultivation to $18.3 \text{ g}_{\text{trehalose}} \text{ g}_{\text{CDW}}^{-1}$

Table 5.4.1. Changes in fermentation performance observed as a result of short-term adaptation of *S. cerevisiae* to lignocellulosic hydrolysate.

Strain	Raw biomass	Pretreatment method	Short term adaptation method	Fermentation method	Change in performance	Reference
Commercial baker's yeast	Spruce	Impregnated with sulfur dioxide, steam pretreated at 215 °C for 5 min.	Fed-batch in bioreactor. At end of feed, medium contained 31% of steam pretreated softwood liquor.	SSF in bioreactor at 5% WIS loading Batch in shake flask. Medium corresponding to 10% WIS content.	$Y_{e/s,24h}$: +53%; $Y_{e/s,48h}$: +10%. $Y_{e/s,4h}$: +55%.	Alkstrawi et al. 2006
Industrial strain KE6-12	Wheat straw	Impregnated with sulfuric acid, steam pretreated at 186 °C for 8 min.	Fed-batch in bioreactor. At end of feed, medium contained 50% of steam pretreated wheat straw liquor.	SSF in bioreactor at 10% WIS loading	$Y_{e/s,120h}$: +65%	Nielsen et al. 2015
Stabilized liquid yeast (Lallemand, Canada)	Sweet sorghum	Ensilage, Reverse Acid Pre-Treatment at 125 °C, for 1 h, enzymatic hydrolysis Ctec3 (Novozymes, Denmark)	Medium: 50% and 75% sweet sorghum hydrolysate either diluted in YPD or water.	Batch in bottles. Medium: non-enzyme hydrolysed, non-diluted sweet sorghum liquor.	All $Y_{e/s} > 95\%$; CO ₂ profiles show adapted cultures finish producing 10 h before non-adapted cultures.	Zhang et al. 2019
Industrial strain KE6-12	Wheat straw	Impregnated with sulfuric acid, steam pretreated at 190 °C for 10 min, enzymatic hydrolysis, Ctec2 (Novozymes, Denmark)	Fed-batch in bioreactor. At end of feed, medium contained 40% of wheat straw hydrolysate.	Batch in shake flask. Medium: 90% wheat straw hydrolysate.	$q_{e,48h}$: +1.6-fold $q_{e,48h}$: +3.6-fold	Paper III
Industrial strain CR01						

during short-term adaptation (**Paper III, Figure 3C**). Therefore, trehalose accumulation cannot be considered a general indicator of short-term adaptation effectiveness.

Only a minor decrease in viability was detected for non-adapted cultures of both strains after 48 h of fermentation of 90% hydrolysate, while adapted cultures showed no decrease in viability under these conditions, (**Paper III, Table 1**). Loss of viability is highly dependent on both the hydrolysate composition and the strain background. However, due to the small differences in observed viability, we cannot conclude that increased viability plays a key role in short-term adaptation in this case. Ultimately, despite the clear physiological differences observed between strains KE6-12 and CR01, both strains showed an improved ethanol productivity and thus both benefit from short-term adaptation. Therefore, it can be concluded that the physiological response of *S. cerevisiae* during short-term adaptation to hydrolysate is strain dependent.

A transcriptomic analysis of cells undergoing short-term adaptation was performed to gain understanding on the metabolic effects of short-term adaptation (**Paper IV**). One aim was to identify the stress response pathways that are important for producing a robust inoculum for hydrolysate fermentation. We had previously observed that the cells must first detoxify the medium by depleting the furfural before growth and fermentation can commence (**Paper II, Table 5**). Furfural generates high intracellular concentrations of ROS (Ask et al. 2013a) and is known to inhibit glycolytic enzymes (Banerjee et al. 1981). Furfural detoxification has been reported to be an important coping strategy of *S. cerevisiae* (Liu 2011). Our transcriptomic study showed that genes *ARI1*, *YLL056C*, *YGL039W*, *YKL107W* and *ADH6*, encoding furfural and/or HMF detoxifying enzymes, were significantly upregulated during short-term adaptation, indicating that this response is beneficial for producing a hydrolysate tolerant inoculum (**Paper IV, Figure 5**).

Further analysis revealed that not many stress-associated transcription factor encoding genes were differentially expressed in short-term adapting cultures compared to non-adapting cultures (**Paper IV, Figure 4**). Among significantly upregulated transcription factors, only Mig3 stood out to have a clear relation to stress (Lewis and Gasch 2012). However, transcription-factor-based regulation is known to occur, not only due to change in expression levels, but also due to translocation of the transcription factors to the nuclei upon stress (Kuge 1997; Gulshan et al. 2005; Swinnen et al. 2017).

The samples for RNA sequencing were taken at four points throughout the feed phase (**Paper IV, Figure 1**). This allowed for analysis of differential expression of genes over time. Additional important stress response pathways could be identified by filtering out genes that were significantly differentially expressed for two or more time points in short-term adapting cultures compared to non-adapting cultures (**Paper IV, Figure 6**). Among the genes that were differentially expressed for two or more time points were *YHK8* and *FLR1*, both encoding multi-drug proton antiporters (Gbelska et al. 2006; Dias et al. 2010). Growth of *YHK8* and *FLR1* overexpressing strains as well as deletion strains in hydrolysate medium, further confirmed their importance in the short-term adaptation process (**Paper IV, Figure**

7). Other interesting genes identified in this way were *THI11* and *THI13*, involved in thiamine metabolism (Wightman and Meacock 2003). Addition of thiamine, together with biotin and pyridoxine, to the propagation was found to improve subsequent hydrolysate fermentation (**Paper II**). Genes involved in biotin metabolism, *BIO3* and *BIO4*, were also found upregulated (**Paper IV, Figure 8**). Thus, these findings provide a better understanding of the molecular mechanisms governing short-term adaptation of *S. cerevisiae* to lignocellulosic hydrolysate and suggest new genetic targets for improving fermentation robustness.

6. Conclusions

The conclusions from this work forms a basis for the better understanding of the molecular mechanisms underlying short-term adaptation of *S. cerevisiae* to lignocellulosic hydrolysate. In addition, the work points out short-term adaptation during propagation is an important tool to improve fermentation performance of lignocellulosic hydrolysates.

The Biolector platform was shown to be capable of reliably monitoring growth profiles of a variety of *S. cerevisiae* strains growing in dark hydrolysate medium in a high throughput manner (**Paper I**). The scattered light method of detecting cell density was clearly not hindered by the coloration of the hydrolysate. This method was proven to work using hydrolysate from two different lignocellulosic biomass sources (wheat straw and corn stover). A case study in which growth of $\Delta Pdr12$ mutants in hydrolysate were screened and compared to their parental strains showed that in order to use this high-throughput cultivation system as a screening tool for bioethanol producers, measuring growth alone is not sufficient and ethanol levels have to be taken into account as well.

Screening studies performed in the Biolector system clarified the role of nutrient addition to propagation of *S. cerevisiae* on subsequent lignocellulose fermentation. Propagation medium was supplemented with several vitamins known to influence LCI tolerance in *S. cerevisiae* both individually and as a mixture (**paper II**). Results showed that the addition of a mixture of the B-vitamins biotin, thiamine and pyridoxine solely to the propagation improves subsequent fermentation performance in hydrolysate by 29% of the theoretical ethanol yield in wheat straw hydrolysate and by 12 % of the theoretical ethanol yield in corn stover hydrolysate. This study also demonstrated that addition of nutrients solely to the propagation of *S. cerevisiae* combined with short-term adaptation is a promising strategy to further improve second generation bioethanol fermentation efficiency.

S. cerevisiae strains CR01 and KE6-12 were both subjected to short-term adaptation to hydrolysate in order to determine physiological parameters indicative of LCI robustness

(**paper III**). The specific ethanol productivity during fermentation increased for both CR01 (3.6-fold) and KE6-12 (1.6-fold) strains after short-term adaptation. The critical specific growth rate of CR01 during propagation was shown to decrease below 0.05 h^{-1} in the presence of 40% (w/w) WSH, whereas this was not observed for KE6-12. Although both strains benefitted from short-term adaptation, cell viability and storage carbohydrate content following short-term adaptation demonstrated that the physiological effect of short-term adaptation is strain dependent.

Transcriptional profiles were compared of adapting and non-adapting cultures during the feed phase of propagation (**paper IV**). Metabolic pathway analysis revealed upregulation of certain genes involved in oxidative stress response genes as well as genes encoding detoxifying enzymes acting on furans. In accordance with results found in **paper II**, several genes in biotin and thiamine metabolism were found to be differentially regulated. Yhk8 and Flr1 were identified as targets for future research as they showed strong upregulation in the short-term adapting cultures compared to the non-adapting cultures. The upregulation of these genes might be involved in the weak acid stress response of *S. cerevisiae* as Yhk8 and Flr1 belong to the *DHA1* multidrug proton antiporter family. Down-regulation of certain transporter encoding genes was also observed. Overall, the sequencing data quality showed good reproducibility among biological replicates in this study. Utilizing multiple time-point samples to identify trends in differential gene expression proved a useful approach to single out relevant genes among those triggered by growth on lignocellulosic hydrolysate.

In conclusion, the work in this thesis has furthered the knowledge of which metabolic processes play a role in the production of a robust inoculum culture through the use of short-term adaptation. It has shown the potential role of B-vitamins, oxidative stress and multidrug resistance transporters in this process and identified them as being points of interest for future research.

7. Future perspectives

S. cerevisiae is widely used in industrial bioprocesses which will always benefit from further optimization. Thus, cellular robustness mechanisms of *S. cerevisiae* that help to combat stress reactions will surely remain a keen topic of research for the foreseeable future. In my thesis work, I have shown that the investigation of real hydrolysates, as opposed to synthetic mixtures of inhibitors, is important to understand the process conditions that *S. cerevisiae* encounters in industrial cultivation.

The Biolector system, employing scattered light detection instead of classical absorption, is suitable for use with dark hydrolysate medium (**paper I**) and thereby opens up possibilities to intensify screening experiments. However, a system such as the Biolector has its limitations. The 1 mL culture volume does not allow for sample taking and therefore, metabolite levels can only be measured post-cultivation. Although the flower-shaped wells of the Biolector have shown to improve oxygenation of the medium compared to classical round wells, it does not allow for real-time control of dissolved oxygen in the medium, such as is possible in bioreactors. Moreover, control of pH or feeding of the culture is also not possible in the Biolector system. Such features would greatly enhance the quality and reproducibility of the obtained data. Commercial systems that implement these features on a small-scale, high-throughput manner are currently available (Velez-Suberbie et al. 2018). However, these systems still rely on conventional absorption methods for cell density monitoring. If scattered light detection could be implemented, this would greatly increase its usefulness in research towards *S. cerevisiae* robustness to lignocellulosic hydrolysates.

Vitamins biotin, thiamine and pyridoxine have shown to be important in the short-term adaptation process (**paper II** and **IV**). It would be of great scientific interest to conduct further investigations into the mechanism of how these vitamins confer robustness. One possibility would be to create overexpression and knockout libraries of genes in biotin, thiamine and pyridoxine metabolism and screen for hydrolysate fermentation performance. This would allow us to identify whether biosynthesis, rather than the mere presence of

these vitamins, improves hydrolysate fermentation. Another aspect that would greatly benefit from further research is the vitamin composition of lignocellulosic hydrolysates. Certain sources of lignocellulosic biomass might contain higher vitamin concentrations than others. High temperatures during pretreatment could lead to degradation of vitamins present in the lignocellulosic biomass. Knowing which vitamins are present in hydrolysates and at what concentrations is essential if we want to perform more extensive studies into the role that nutrient addition plays during lignocellulosic cultivation.

Investigation of the strain-dependence nature of short-term adaptation (**paper III**) would benefit greatly from screening more *S. cerevisiae* strains. The method described in **paper I** would allow these screenings to be done in a more desirable time frame than using the conventional methods for monitoring of hydrolysate cultivations. Examples of strain backgrounds that would be interesting for further studies are industrial strains (Ethanol Red, DGI 342, PE-2, KE6-12), isolates from other industrial processes (i.e., cachaça production (da Conceição et al. 2015), distillery waste streams (Pandey et al. 2019) or others (Pereira et al. 2014)), and even laboratory strains for reference to conventional literature on LCI tolerance.

Transcriptomics revealed the importance of several genes that have thus far not been extensively studied in *S. cerevisiae* (**paper IV**). It would be of great value to further investigate these proteins and their role in short-term adaptation. In **paper IV**, data showed that YHK8 and FLR1 stood out in their differential expression pattern. Although Yhk8 and Flr1 are both known to be members of the multidrug proton antiporter DHA1 family (Gbelska et al. 2006; Dias et al. 2010), details of their mechanism of activity remain unclear. Yeast multidrug resistance (MDR) transporters are generally thought to actively transport a wide variety of unrelated drugs and other xenobiotic compounds from the cytosol to the outer medium, thus providing protection from their toxicity. However, this model has been brought into question and evidence supports the idea that some of these transporters might have a specific substrate (Sá-Correia et al. 2009). Other proteins, currently of unknown function, that were found to be of significance in our dataset were Aad4, Cis1, Dpa1, Hbn1, YLL056Cp, YMR315Wp and YLR108Cp. Enzymatic activity assays as well as overexpression experiments combined with physiological characterization of such overexpression strains would be a good first step in elucidating the mechanisms by which these proteins improve robustness of *S. cerevisiae* to hydrolysate stress.

Short-term adaptation has been shown to have great potential for improving second generation bioethanol production with minimal impact on operational costs. Better understanding of the mechanisms underlying short-term adaptation can inform metabolic engineering approaches but can also be used to optimize propagation procedures in industry.

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